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Establishing Relationships Between DNA Repair and Transcriptional Mutagenesis of Non-Bulky Base Damage in *Escherichia coli*

By

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Doctor of Philosophy

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DNA damage occurs continuously, but faithful replication and transcription are essential for maintaining cell viability. Generally, cells in nature do not divide or replicate DNA often, thus, it is important to consider the outcome of RNA polymerase (RNAP) encounters with DNA damage. Base damage in the DNA can affect transcriptional fidelity, leading to production of mutant mRNA and protein in a process termed transcriptional mutagenesis (TM). It was of interest to determine how DNA repair pathways are involved in the process of TM. The contributions of base excision repair (BER), transcription-coupled repair (TCR), and nucleotide excision repair (NER) to repair of two non-bulky lesions, 8-oxoguanine and uracil, were examined *in vivo* using a luciferase-based reporter assay in *Escherichia coli* under non-growth conditions. We found that both TCR and NER are utilized by *E. coli* to repair 8-oxoguanine and uracil. We also found that TCR can utilize components of either pathway for lesion removal. These findings indicate a dynamic flexibility of DNA repair pathways in the removal of non-bulky DNA lesions in prokaryotes, and reveal their respective contributions to the repair of 8-oxoguanine and uracil *in vivo*. It was also of interest to determine the consequences of incomplete BER, which results in abasic (AP) sites and strand breaks, on the process of TM. These lesions also frequently occur spontaneously, so RNAP could often encounter these non-coding structures *in vivo*. We were able to demonstrate that RNAP is capable of bypassing AP sites and strand breaks in *E. coli* resulting in TM through adenine incorporation in nascent mRNA. Elimination of the enzymes that process AP sites and strand breaks further increases TM. TM has many potential biological consequences, including adaptive mutagenesis (directed evolution) in bacteria, as well as cancer and neurodegenerative diseases in humans. Discerning the contributions of DNA repair to the process of TM can aid in our understanding of the initiation and progression of important human diseases.

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CHAPTER 1

GENERAL INTRODUCTION

DNA is the molecule responsible for carrying all of the genetic information for an organism, and yet it is chemically unstable. DNA can be modified through several mechanisms, including hydrolysis, oxidation and methylation (Figure 1), resulting in DNA damage (1). In dividing cells, unrepaired damage can direct the incorporation of incorrect bases into the newly synthesized DNA during replication. In a laboratory setting, cells are nurtured and given all of the media components necessary to grow and divide often. Therefore, studying mutagenesis during DNA replication was appropriate, as DNA polymerase encountered DNA damage frequently. But, most cells in nature are not undergoing continuous rounds of replication and dividing constantly (2). It has been demonstrated that *E. coli* divide infrequently, only doubling once about every 40 hours in mammalian colons, and not dividing at all once excreted outside of a body (3). Also, many organ systems in multicellular organisms contain terminally differentiated cells that do not undergo DNA replication. Thus, vitality of most cells is largely dependent on the fidelity of transcription and translation, and not replication. Encounters of RNA polymerase (RNAP) with DNA damage can result in one of three outcomes: (i) RNAP blockage and transcriptional arrest, (ii) initiation of transcription-coupled repair (TCR) and removal of the lesion, or (iii) RNAP bypass of the lesion resulting in transcriptional mutagenesis (TM).

Transcriptional Mutagenesis

TM is the process by which RNAP bypass of DNA damage with altered base pairing properties results in generation of a mutant transcript via incorporation of an incorrect ribonucleotide into the nascent mRNA during transcription (Figure 2). TM was

first described in 1993 (4) and progress on elucidating its mechanism and biological impact was recently reviewed in 2006 (5).

***In vitro* studies on TM.** Most of what is known about TM has derived from *in vitro* studies with purified components, resulting in a decent understanding of which lesions can be subject to RNAP bypass. Many groups have examined the bypass efficiency and have also sequenced the transcripts that resulted from bypass, allowing for a determination of the mutagenic potential of many base lesions. Table 1 summarizes the current TM data in the literature and includes lesion bypass efficiency, available information about RNAP insertion events, and the type of resultant mutation.

In vitro RNAP can readily bypass smaller base lesions which would typically be repaired by base excision repair (BER, illustrated in Figure 3). These include the cytosine deamination product uracil (4, 6-11), the methylated base O^6-methyguanine (9, 12) and several products of oxidative base damage including 8-oxoguanine (6, 8, 9, 13-17), dihydrouracil (7, 18), 8-oxoadenine (17), 2-hydroxyadenine (17), 5-hydroxyuracil (13), 5-hydroxycytosine (15), and thymine glycol (13, 15, 17, 19, 20). The miscoding properties of modified bases can vary drastically (Table 1). Cytosine-derived products such as uracil and 5-hydroxyuracil are almost always miscoding, both directing incorporation of adenine into the mRNA (4, 6, 7, 9-11, 13)—though one study reported that mammalian RNAP II could incorporate both adenine and guanine opposite uracil (6). Many lesions demonstrate both mutagenic and non-mutagenic events in mRNA (Table 1). 8-oxoguanine will direct both cytosine and adenine insertion (6, 9, 13, 14, 16, 17), while both adenine and guanine have been observed in the mRNA opposite dihydrouracil (7, 18). O^6-methylguanine shows differing rates of uracil and cytosine incorporation into

the mRNA depending on the RNAP examined (9, 12), and 8-oxoadenine is more often non-mutagenic, instructing uracil insertion, but there is some amount of adenine observed as well (17). Additionally, both thymine glycol and 2-hydroxyadenine are not miscoding at all (non-mutagenic), directing incorporation of adenine and uracil respectively (13, 17).

In addition to damaged bases, downstream intermediates of their repair might also be encountered by the transcriptional machinery (Figure 3). Abasic sites and the abasic site analog tetrahydrofuran are both readily bypassed by RNAP (4, 6, 8, 10, 14, 21), although abasic sites that have been modified by oxidation are bypassed much less efficiently (22). Prokaryotic RNAPs demonstrated a preference for inserting adenine opposite an abasic site (4, 10), while mammalian RNAP II has demonstrated a preference for cytosine (6). Also, guanine incorporation was observed opposite tetrahydrofuran, an AP site analog, with phage RNAP (14). As will be discussed in detail below, abasic sites can be generated by several mechanisms, making it difficult to predict whether these incorporation events would be mutagenic. It should be noted that guanine depurination is a much more frequent event giving rise to spontaneous abasic site formation compared to adenine depurination or depyrimidination (23), so most often both the adenine and guanine events observed for prokaryotic RNAPs would result in mutagenesis.

Surprisingly, a number of bulky DNA lesions can be bypassed by RNAP as well, although they typically have low bypass efficiencies (Table 1). 5-guanidino-4-nitroimidazole, an oxidation product resulting from a direct reaction of peroxynitrite with guanine, is bypassed efficiently by T7 RNAP, resulting in insertion of different bases, but non-mutagenic cytosine is the most frequent (24). Mammalian RNAP II does not bypass this lesion as well as T7 RNAP, and it only directs incorporation of the non-mutagenic

cytosine (24). Guanine C-8 aminofluorene (AF) and acetylaminofluorine (AAF) adducts are both bypassed by RNAP in a non-mutagenic manner, with reduced efficiency for the bulkier lesion (AAF) (14, 25). N^6-benzo[a]pyrene diol epoxide adducts of both guanine and adenine can be bypassed by phage RNAP (26, 27). Adenine adducts resulted in misincorporation of either adenine or guanine (27), while transcripts resulting from guanine adduct bypass resulted in non-mutagenic cytosine incorporation (26). It should be noted that in the guanine study the authors found when RNAP stalled at the lesion, resulting in truncated transcripts, the transcripts contained mutagenic nucleotide insertion events, suggesting that the RNAP arrest may result from an inability to extend the transcript after incorporation of an incorrect nucleotide into the mRNA. Recently 8,5'-cyclo-2'-deoxyadenosine, a bulky oxidation product of guanine, has been shown to be occasionally bypassed by RNAP II, resulting in either non-mutagenic uracil or a deletion in the mRNA (28). The most surprising report is that cyclobutane pyrimidine dimers, bulky lesions resulting from ultraviolet light, can be bypassed by mammalian RNAP II, although very rarely, resulting in large deletions (28). Cyclobutane pyrimidine dimers are well characterized as substrates for transcription-coupled repair (described below) primarily because of their ability to arrest RNAP at the site of the lesion (29), making the RNAP bypass of these lesions particularly noteworthy.

It is important to note that these *in vitro* studies have not been limited to RNAP from any particular species. Studies have utilized RNAP from phage (4, 7, 10-12, 14, 16, 19-22, 24, 26, 27, 30, 31), *E. coli* (4, 8-10, 18, 30, 32, 33) and mammals (6, 12, 13, 15-17, 19-22, 24, 25, 28, 34, 35) demonstrating the potential for TM in a variety of organisms.

***In vivo* studies on TM.** While lesions that are bypassed by RNAP *in vitro* are likely to also be bypassed by RNAP *in vivo*, very few studies have been conducted in living cells (32-36). Most groups have taken advantage of a luciferase reporter gene system for the measurement of TM *in vivo*. This assay is described in detail below. It has been successfully used to demonstrate TM caused by uracil and 8-oxoguanine in *E. coli* under non-growth conditions (32, 33), and an adaptation of the assay has also been used to examine 8-oxoguanine bypass by RNAP in mammalian cells (34, 36). Additionally, TM caused by 8-oxoguanine in mouse embryonic fibroblasts in the *RAS* proto-oncogene can occur in a manner that leads to a constitutively active, mutant H-Ras protein (35). Activating mutations of *RAS* have been found in nearly one third of all human cancers (37) and leads to increased activation of the MAP kinase pathway (38). TM in *RAS* led to activation of this signaling cascade, assayed by measuring phosphorylation status of Erk, demonstrating for the first time that TM can have biologically relevant consequences (35).

In all of the *in vivo* studies it was observed that elimination of DNA repair enzymes, described in detail below, could increase TM. In mammalian cells, elimination of BER (Ogg1) (34, 35) or mismatch repair (hMsh2/hMsh6) (34) led to a significant increase in TM caused by 8-oxoguanine, whereas elimination of TCR (Csb) had no effect (34, 35). For uracil-mediated TM in bacteria, elimination of BER (Ung, Mug) led to a significant increase in TM (32, 33). Additionally, 8-oxoguanine-mediated TM in bacteria was exacerbated when BER (MutM), mismatch repair (MutS), or TCR (Mfd) were disrupted (32). These results demonstrate the importance of understanding the contributions of DNA repair pathways to TM *in vivo*.

DNA Repair Pathways

Base Excision Repair. BER is thought to be primarily responsible for the repair of minimally distorting and non-bulky lesions such as oxidized or ring-saturated bases, alkylated, or deaminated bases (39). As shown in Figure 3, BER is initiated by one of several damage-specific DNA N-glycosylases, which facilitates removal of the lesion through cleavage of the N-glycosidic bond that attaches the damaged base to the sugar-phosphate backbone of the DNA. Because each DNA N-glycosylase recognizes a specific, limited set of base damages, there are multiple glycosylase enzymes in every organism. *E. coli* has eight glycosylases while humans have twelve (Table 2) (39). Glycosylases can be either monofunctional or bifunctional. Monofunctional glycosylases, which include Tag, AlkA, Ung, Mug, and MutY in *E. coli*, catalyze the removal of the damaged base, leaving an abasic site (apurinic/apyrimidinic or AP site). Bifunctional DNA N-glycosylase/AP lyases, such as MutM (Fpg), Nth, and Nei in *E. coli*, not only remove the damaged base, but also possess a 3′-AP lyase activity which incises the phosphodiester bond at the 3′ side of the deoxyribose via β-elimination, leaving a single strand break (SSB) with 3′-phospho-α,β-unsaturated aldehyde (3′-PUA) and 5′-phosphate (5′-P) ends (Detailed in Figure 4). MutM and Nei can further carry out δ-elimination, resulting in the removal of the unsaturated aldehyde moiety and generation of 3′-phosphate terminus (3′-P) (39). Many homologs of the *E. coli* glycosylases are conserved from *E. coli* to humans (Table 2), including the enzymes relevant for these studies, Ung and MutM. Ung, which catalyzes the removal of uracil from DNA, has a human homolog UNG (40, 41). MutM, which catalyzes the removal of oxidized and

ring-opened purines from DNA, has both an ortholog and a functional homolog in humans in the forms of NEIL1 and OGG1, respectively (41).

AP sites generated as a result of glycosylase activity or spontaneous base loss are mutagenic, and therefore must be rapidly removed from the DNA (42). They are processed via strand cleavage by an AP endonuclease or an AP lyase (Figures 3, 4). AP lyase activity has already been discussed as an additional activity of the glycosylases MutM, Nth, and Nei. AP endonucleases process AP sites by cleaving the phosphodiester bond at the 5′ side of the AP site, generating 5′-deoxyribose phosphate (5′-dRP) and 3′-hydroxyl termini (3′-OH) (Figure 4) (39). There are two AP endonucleases in *E. coli*, Xth (exonuclease III) and Nfo (endonuclease IV).

Whether the AP site is handled by an AP endonuclease or an AP lyase, the resulting DNA ends need further processing, as the gaps left by these enzymes are not appropriate for polymerase gap filling and ligation, and they can also be mutagenic (42). A 5′-dRP can be effectively removed in *E. coli* by Fpg, Nei, or RecJ or by the 5′-to-3′ exonuclease activity of Pol I (39) (Figure 3, 4). The *E. coli* AP endonucleases can effectively process both β- and β,δ-elimination products through 3′-phosphodiesterase and 3′-phosphatase activities, respectively (Figure 3, 4) (43-45). Xth also possesses a 3′-to-5′ exonuclease activity that facilitates removal of more than one nucleotide. Additionally, nucleoside diphosphate kinase (NDK) has been shown to have AP-lyase, 3′-phosphodiesterase, 3′-phosphatase, and 3′-to-5′ exonuclease activity, although it is unclear whether this enzyme plays a role in BER (46, 47).

Following end trimming, the gap produced is subsequently filled in by a DNA polymerase through one of the BER sub-pathways: short-patch (SP), which involves

replacement of a single nucleotide, or long-patch (LP), that involves replacement of several nucleotides (Figure 3). If end processing has been completed successfully, resulting in a 3′-OH and a 5′-P, *E. coli* DNA Polymerase I (Pol I) can carry out SP-BER by inserting one nucleotide in the gap, and ligase I can seal the nick, completing repair and restoring the original DNA sequence (39). If a 5′-dRP residue is not removed prior to repair synthesis, LP-BER is carried out via strand displacement by Pol I (48), leading to the addition of two to eight nucleotides (49), with the displaced strand eventually being cleaved by Pol I 5′-to-3′ exonuclease activity (50, 51).

BER is highly conserved from bacteria to humans. All of the individual steps of BER are conserved, though additional enzymes have evolved in higher organisms (Reviewed in Krwawicz et al., (39)). Table 2 is a summary of all of the enzymes involved in each step of BER, including the specific names of the human and *E. coli* genes.

Nucleotide Excision Repair. Nucleotide excision repair (NER) is a process by which damaged bases are enzymatically excised from the genome embedded in oligonucleotide fragments, rather than as free bases as in BER (52). While the number of proteins involved in NER in prokaryotes is modest compared to eukaryotes, the general process of incisions flanking the damaged base and excision of an oligonucleotide fragment containing the damage is conserved from bacteria to mammals (Table 3) (29). NER is thought to be involved primarily in the repair of large, distortive DNA adducts (53), however NER has also been shown to be capable of removing lesions not generally considered to be distortive (54-59).

NER that acts on the bulk of the genome, and that is not linked to transcription, is sometimes termed global genome repair, or GGR. The first steps of GGR (damage recognition, 3'-incision and 5'-incision flanking the lesion) are carried out in *E. coli* by an ensemble of three proteins, UvrA, UvrB, and UvrC, with a complex of UvrA and UvrB acting to initiate NER through lesion recognition (Figure 5). Purified UvrA associates with UvrB to form a $(UvrA)_2UvrB$ complex *in vitro* at physiological concentrations (60), although there is evidence that UvrB acts as a dimer as opposed to a monomer (61). The interaction of UvrA and UvrB is strictly dependent upon hydrolysis of ATP (53). Once the matchmaker role of $(UvrA)_2$ has been accomplished (62), it will dissociate, leaving a UvrB-DNA complex (60, 63-66). UvrC can associate with the UvrB-DNA complex, sequentially incising the DNA 3′ and 5′ to the damage (Figure 5) (63, 67). While the incision 5′ to the damage typically occurs at the eighth phosphodiester bond from the damage (60), the site of the 3′ incision is more variable, occurring at either the fourth or fifth phosphodiester bond from the damage (68-72). DNA sequence context can affect these incision sites (73).

Following incision of the phosphodiester backbone around the damage, it is necessary to remove the oligonucleotide containing the damage and fill in the resulting gap. The excision step removes the oligonucleotide containing the damage via the action of the *uvrD*-encoded DNA helicase II (Figure 5). DNA helicase II is absolutely required for the excision of the oligonucleotide and release of UvrC, and other *E. coli* helicases are unable to substitute for this activity (74). As UvrB has yet to be released at this stage, it is possible that UvrB helps recruit DNA helicase II to the incised DNA (74). The small

gap created is then filled in by DNA polymerase I and sealed by ligase, leaving fully repaired DNA (75).

Transcription-Coupled Repair. TCR specifically targets lesions on the transcribed strand of active genes which affect the progression of RNA polymerase (RNAP) during transcription. This pathway is found in most bacteria and many eukaryotes, but has yet to be found in archaea (76). TCR is generally considered to be a subpathway of NER, using an alternative initiation step to recruit repair proteins to a stalled RNAP.

The TCR mechanism is well understood in bacteria and is beginning to be better understood in higher organisms as well (29). In *E. coli*, when RNAP encounters a lesion in the DNA that causes it to stall, it is bound by the transcription-repair coupling factor, Mfd, which releases RNAP and recruits UvrA (Figure 5). From this point, repair proceeds through NER in a mechanism indistinguishable from GGR (Figure 5).

In *E. coli* TCR, Mfd displaces RNAP from the lesion through use of its tandem RecA-like domains that contain helicase motifs (77). The DNA-dependent ATPase activity allows Mfd to translocate along the DNA, pushing RNAP forward. If a stalled RNAP has also backtracked, the 3′ end of the transcript will become repositioned in the active site and transcription can resume (78). However, if there is a lesion blocking forward progression by the RNAP, Mfd "pushing" can force release of the RNAP from the DNA (79). At this point, the RNA product is likely destroyed once the RNAP-RNA-DNA complex is disrupted (80). Therefore transcription will have to reinitiate from the promoter after repair has been completed.

As stated above, TCR is conserved from bacteria to humans. Similar to other DNA repair pathways, the basic mechanism of repair is the same in all organisms, though the complexity increases in higher organisms (Table 3) (29). In mammals the transcription-repair coupling factor is Csb, which is named for the disease caused by its deficiency, Cockayne Syndrome (81). Like Mfd, it also contains a DNA-dependent ATPase (77). Csb may also push the RNAP forward, but dissociation of the RNAP from the DNA was not shown *in vitro* (82). This makes sense when one considers that mammalian genes, which contain introns, are often significantly larger than those of bacteria, and the cost of having to reinitiate transcription after repair could be too great. Indeed, the largest human gene, dystrophin, is 2.8 MB long and requires approximately 16 hours to transcribe (83). Like bacteria, after recruitment of Csb and other factors to the lesion, repair proceeds through the NER pathway as it would for GGR. The GGR initiating factors XPC and DNA damage binding-2 (DDB2) are dispensable for TCR because Csb facilitates the necessary recognition step (29).

Mismatch Repair. Though not specifically examined in this dissertation work, mismatch repair (MMR) has been demonstrated to be important in the prevention of transcriptional mutagenesis caused by 8-oxoguanine in both *E. coli* (32) and mammals (34). The MMR pathway is important for increasing the fidelity of replication through the removal of replication errors which are not corrected by the DNA polymerase itself. Error rates during DNA synthesis are around 10^{-6} to 10^{-8} for the replicative polymerases. High fidelity likely results from a 10^4 to 10^6 selectivity by the polymerases for selecting the correct nucleotide instead of an incorrect nucleotide and an additional removal of 90-99.9% of the remaining errors by intrinsic proofreading exonuclease activity of the DNA

polymerases (84). MMR can further increase replication fidelity by 50 to 1000-fold, so that actual spontaneous mutation rates for prokaryotic and eukaryotic microbial genomes are only around one mutation per 10^9 to 10^{10} base pairs per cell division (85, 86). Additionally, several types of DNA damage have been demonstrated to be processed by MMR including O^6-methylguanine (87-91), 8-oxoguanine (92, 93), carcinogen adducts (94), UV photo products (95-97), and cisplatin adducts (90, 98, 99). MMR is well conserved between *E. coli* and humans (85), and MMR inactivation in humans causes hereditary nonpolyposis colon cancer (100-103) and has been implicated in the development of sporadic cancers in a variety of other tissues as well (104-106).

In *E. coli* MMR initiates with mismatched base pair recognition by MutS, which exists as either a dimer or tetramer (107-111). Next, MutL is recruited to the site of the mismatch in a MutS- and ATP-dependent manner (112-116). Formation of the MutS-MutL-heteroduplex ternary complex is sufficient to recruit MutH, an endonuclease specific for unmodified GATC sequences in *E. coli*. MutH incises 5′ to the G on the unmethylated strand of a hemimethylated GATC sequence in a mismatch-, ATP-, MutS- and MutL-dependent manner (117, 118). It is because of this strand-specific cleavage by MutH that MMR is able to differentiate between the template strand and the newly synthesized DNA strand following replication. Interestingly, this strand discrimination is possible even if the closest GATC sequence is 1000 base pairs away from the site of the mismatch (119), and MutH can incise either 5′ or 3′ to the site of the mismatch, indicating that mismatch repair has no preferred polarity (120, 121).

Formation of the MutS-MutL-heteroduplex complex is also sufficient to activate methyl-directed excision system which includes DNA helicase II (encoded by *uvrD*) and

several single-strand specific exonucleases. Helix unwinding begins at the site of the strand break and is heavily biased in the direction of the mismatch (122), likely due to coordination with MutL (122-127). As the DNA is unwound, the exonucleases can excise the nicked (unmethylated) DNA strand. When the MutH incision is 5′ to the mismatch, the exonucleases ExoVII and RecJ , both of which can excise DNA with 5′ to 3′ polarity, are responsible strand excision (128-131). When the MutH incision is 3′ to the mismatch, ExoI, ExoVII, and ExoX, all of which can excise DNA with 3′ to 5′ polarity, are responsible for strand excision (132-136). Following excision, SSB stabilizes the remaining strand until gap filling by DNA polymerase III and nick sealing by ligase. The use of DNA polymerase III for gap filling includes a requirement for the β clamp processivity factor, which has been shown to physically interact with MutS (137), and the γ complex of the DNA polymerase III holoenzyme for loading of the β clamp (138).

The DNA Lesions Examined in these Studies

DNA can be damaged in a variety of ways, resulting in a wide spectrum of DNA lesions. These lesions do not occur with equal frequency in the DNA, so it is important to select lesions for study that are frequently occurring but also biologically relevant. In these studies we have utilized 8-oxoguanine, uracil, abasic sites and strand breaks, all described in detail below.

8-oxoguanine. Reactive oxygen species (ROS) are constantly generated as byproducts of aerobic metabolism and exposure to various natural and synthetic agents. ROS include hydrogen peroxide (H_2O_2), hydroxyl radical (·OH) and superoxide ($\cdot O_2^-$)

(139, 140). Although ROS can damage all intracellular macromolecules, including proteins, lipids, carbohydrates and RNA (141, 142), DNA damage is of particular importance for mutagenesis (143-146). In addition to base loss and strand breaks caused by attacks at the sugar moiety (147, 148), there are over 80 known base damage products caused by ROS (149). Some susceptible sites are shown in Figure 1b.

One of the most common, biologically important oxidative lesions is 7,8-dihydro-8-oxoguanine, or 8-oxoguanine (Figure 6a). It is estimated that as many as 1,000-2,000 8-oxoguanine lesions are deposited into the mammalian genome in every cell, each day (150). 8-oxoguanine is mutagenic as it can assume a *syn* conformation, readily base-pairing with adenine (Figure 6a) and resulting in transversion mutations during replication (151-153). Additionally, 8-oxoguanine can be bypassed by RNAP both *in vitro* and *in vivo*, resulting in cytosine or adenine incorporations opposite the lesion, as well as -1 deletions (6, 8, 9, 13-17, 32, 34-36).

Uracil. Uracil is a base normally found in RNA, but it can occur inappropriately in DNA through cytosine deamination (Figures 1b, 6b). While deamination of cytosine occurs spontaneously (154), it can also be promoted by many factors, including the formation of UV radiation-induced cyclobutane pyrimidine dimers (155, 156), some intercalating agents (157), or the positioning of a mismatched or alkylated base opposite cytosine (158, 159). Deamination can be chemically induced by nitrous acid (160) and sodium bisulfite (161). Nitrous acid can also cause deamination of adenine and guanine (Figure 1b) (162) or strand cross-links (163), while sodium bisulfite specifically promotes deamination of cytosine (161). Additionally, uracil can be erroneously incorporated into

DNA during replication (164-172), with the extent of this incorporation dependent upon the size of the intracellular dUTP pool (170).

The occurrence of uracil in the DNA is less frequent than oxidative DNA damage, with only approximately 100 events per cell, per day in the mammalian genome (150). Nonetheless, it is of biological importance as *E. coli* and human cells unable to remove uracil from their DNA show increased mutation rates (173, 174). Additionally, a significant replacement of thymine by uracil in DNA can affect the recognition of nucleotide sequences by DNA-binding proteins and enzymes (175). Importantly, uracil can be bypassed by RNAP both *in vitro* and *in vivo*, resulting in transcriptional mutagenesis via adenine incorporation in the mRNA (4, 6-11, 32, 33).

Abasic sites. Bases can be lost from nucleic acids through cleavage of the N-glycosidic bond resulting in an intact backbone and abasic site, also called an apurinic or apyrimidinic (AP) site (Figures 4, 6c). The deoxyribose sugar at the AP site is a highly unstable carboxonium ion that undergoes rapid hydrolysis to a diastereomeric mixture of 2-deoxy-α-D-ribose and 2-deoxy-β-D-ribose (Figure 6c) (23). Additionally, the 2-deoxy-D-ribose anomers may also exist in the ring-opened, aldehyde configuration (Figure 6c) (176).

AP sites occur continuously in the DNA, whether spontaneously, through exposure to chemicals, or through enzymatic activity of monofunctional DNA N-glycosylases, discussed above. Spontaneous depurination occurs approximately 100 times more frequently than spontaneous depyrimidination (177, 178), and it has been estimated that there are 10,000 spontaneous depurination events per cell, per day in the mammalian genome (162, 179).

Chemical induction of AP sites can occur through several mechanisms. Acid-catalyzed hydrolysis occurs via protonation of the N^7 atom of guanine and adenine and/or the N^3 atom of adenine (Figure 1b) (180-185). Ethylating and methylating agents can generate AP sites through labilization of the N-glycosidic bond following alkylation of the N^3 or N^7 atoms of both purines (184, 186-191) or of the O^2 atoms of the pyrimidines (Figure 1b) (192).

AP sites are of great biological importance. All of the combined mechanisms of AP site formation result in an estimated 18,000 AP sites per cell, per day in the mammalian genome (150). It is known that *in vitro* DNA polymerases bypassing AP sites will preferentially incorporate adenine opposite the AP site (193, 194). Given the drastic difference in the rate of depurination versus depyrimidination, adenine incorporation opposite an AP site will most often be mutagenic. Additionally, *in vitro* analysis has revealed that AP sites and the stable AP site analog tetrahydrofuran (Figure 6d) can be bypassed by RNAP, resulting in adenine, and possibly cytosine, incorporation in the mRNA (4, 6, 8, 10, 14, 21).

Single strand breaks. Single strand breaks (SSBs) can occur through many mechanisms including ionizing radiation (195, 196), exposure to ROS (143), exposure to chemicals (197), or through the action of DNA repair enzymes (52, 198). The SSBs that result from DNA damage processing by BER enzymes are of particular interest as the resulting DNA strand ends can vary widely (Figure 4). As discussed above, AP endonuclease activity results in a break with a 3′-OH and a 5′-dRP (198). AP lyase activity results in a break with a 5′-P and either a 3′-PUA or 3′-P (198). Still another possible SSB structure resulting from BER activity is that which results after the above

blocking groups are removed, a 3′-OH and 5′-P (Figure 4) (198). It seems that SSBs would be bypassed poorly by RNAP. However, *in vitro* studies have demonstrated bypass by phage and *E. coli* RNAP to varying degrees, dependent upon the RNAP, and the break ends (4, 10, 11, 30) (Table 1).

***Measuring Transcriptional Mutagenesis* in vivo**

To conduct TM studies *in vivo* requires several tools. The first is a method of maintaining the cells in a non-growth state so that results are not confounded by replication events. The second tool is a reliable method of introducing the particular DNA damage of interest into a known location of a transcribed gene. Finally, it is necessary to have a reporter assay that is informative at the level of both the mRNA as well as the final protein product. The specific tools used in bacterial TM studies are described below.

Growth restriction with novobiocin. The interpretation of TM results could easily be confounded by replication events also contributing to the mutagenesis being measured. Thus, it is important to keep cells in a non-growth state for the duration of an experiment. This is achieved in bacteria through use of the DNA gyrase inhibitor novobiocin, which renders *E. coli* incapable of DNA replication but does not affect transcription (33). Novobiocin provides the additional advantage of mimicking natural non-growth states of cells, as microarray-based gene expression studies demonstrate that many of the classes of genes that are upregulated during recovery from stationary phase in *E. coli* correspond to those that are downregulated during treatment with novobiocin (199).

A reliable method for generation of damage-containing construct. It is often desirable, when studying mutagenesis, to take advantage of a specific DNA lesion in a targeted location of a gene so that results are not complicated by sequence context, or by having a diverse mixture of lesions that could result from random mutagenesis by a DNA damaging agent. The challenge is to find a reliable method that provides high yields of pure, damage-containing construct. Several years ago, our lab developed such a method (Figure 7) (200). The plasmids for luciferase expression (described in detail below) (32) contain an f1 origin of replication in the correct orientation to direct the packaging by bacteriophage of a circular single-stranded DNA (ssDNA) moiety containing the non-template strand of DNA (Figure 7a). This ssDNA can then be isolated and used as the substrate in a polymerase/ligase reaction using a damage-containing oligonucleotide to prime the synthesis of the template strand, resulting in a specific DNA lesion in a targeted location (Figure 7b). The products of this reaction can then be resolved on an agarose gel containing ethidium bromide. Ethidium bromide causes supercoiling of the covalently closed double-stranded DNA (dsDNA), which increases its mobility in the agarose gel and allows for separation from the ssDNA substrate as well as other dsDNA forms (Figure 7c). The desired covalently closed dsDNA can then be excised from the gel and purified. This DNA is hemimethylated, and *in vitro* methylation of the newly synthesized strand can be done using commercially available DAM methylase. The presence of the lesion can be confirmed using commercially available BER N-glycosylase and AP endonuclease enzymes which will remove the damaged base and nick the backbone, causing the construct to run slower in a gel containing ethidium

bromide than covalently closed, unnicked DNA that does not contain the lesion of interest.

Transcriptional Mutagenesis Luciferase Assay System for measuring levels of TM in mutant strains. The TM luciferase assay system (TM-LAS) (Figure 8) in growth-restricted *E. coli* is an extremely useful method because it provides a highly sensitive, real-time indicator of TM and DNA lesion repair *in vivo* by allowing measurement of either the RNA product (via cDNA sequencing) or the translated, enzymatically active protein product (luciferase activity assay) (32). Constructs containing the lesion of interest are transformed into repair-proficient and -deficient *E. coli* via electroporation and immediately incubated in LB medium containing novobiocin to restrict replication (Figure 8a). Luciferase gene expression can be induced by the addition of IPTG to the medium. At different time points following luciferase induction, aliquots of cells can be lysed and assayed for luciferase activity, giving a measurement of protein activity. Additionally, RNA can be isolated, subjected to RT-PCR and subcloned into pUC18 for transcript sequencing. This allows for a readout of the mRNA that results from RNAP bypass of a lesion of interest.

For our studies, codon 445 of the luciferase gene was the target codon. In a control construct containing the wild-type sequence of luciferase (Normal/Normal), codon 445 encodes a lysine and should result in the production of active (wild-type) luciferase protein (Figure 8b). In a Stop/Stop construct, the Lys445 codon (5'-AAA-3') was replaced by a STOP codon (5'-TAA-3') and should result in the production of truncated, inactive luciferase protein (201). A Lesion/Stop construct (in which the lesion is placed opposite the T of the STOP codon) will produce full-length, active luciferase

only when RNAP bypasses the lesion and inserts a nucleotide other than uracil opposite the lesion. In the event of repair in plasmid DNA by the bacterial DNA repair systems, thymine in the non-template strand will specify adenine incorporation on the template strand during DNA repair synthesis. Adenine will code for uracil in the mRNA transcript, resulting in a Stop codon and a truncated, inactive luciferase (201). Therefore, a luciferase activity level (RLUs) produced from the Lesion/Stop construct that is greater than the Stop/Stop control construct will clearly indicate RNAP bypass. The sensitivity of the TM-LAS allows for detection of even small levels of RNAP bypass, an advantage that has not been available with *in vitro* assays.

Additionally, RT-PCR allows for examination of the transcripts that result from RNAP bypass of DNA lesions, through conversion of the mRNA to cDNA, which can then be subcloned and sequenced (Figure 8). cDNA sequencing provides information about which ribonucleotide is incorporated opposite damaged DNA, as well as whether or not the transcripts contain nucleotide deletions (frameshifts). Combined with the TM-LAS, these tools provide a comprehensive view of TM *in vivo*.

Can DNA repair pathways impact transcriptional mutagenesis* in vivo*?

In non-dividing cell populations, the contribution of TM to the mutant protein load and resulting cellular phenotype could outweigh the contribution of mutagenesis that occurs during replication. Genomic integrity is maintained by DNA repair pathways, described in detail above, which can remove damage from DNA, and restore the original nucleotide sequence. Previous work from our laboratory demonstrated that, in *E. coli*, uracil-mediated TM is attenuated by BER, and TM caused by 8-oxoguanine is

significantly increased in the absence of BER, TCR, or mismatch repair (32). The goal of this dissertation is to further elucidate the contributions of DNA repair pathways to the process of TM *in vivo*. The work in this dissertation addresses several questions. (1) Can TCR initiate repair on non-bulky base lesions *in vivo*, specifically 8-oxoguanine and uracil? (2) As non-bulky base lesions are frequently repaired by BER, would TCR of non-bulky base lesions in *E. coli* proceed through the NER pathway, or would TCR take advantage of the lesion specificity of BER? (3) Can repair intermediates such as strand breaks and AP sites be bypassed by RNAP *in vivo*? (4) If repair intermediates are bypassed by RNAP, is the bypass mutagenic, leading to TM? By answering these questions, we provide insight into how DNA repair affects TM *in vivo*. DNA repair pathways can prevent TM through the removal of lesions from the DNA before RNAP can encounter them. Alternatively, DNA repair pathways may contribute to TM through the introduction of repair intermediates to the DNA that themselves cause TM. TM could have many important biological implications, including roles in neurodegeneration and cancer (5). A better understanding of the mechanism of TM and the contribution of DNA repair to this process could have implications in the treatment and prevention of human disease.

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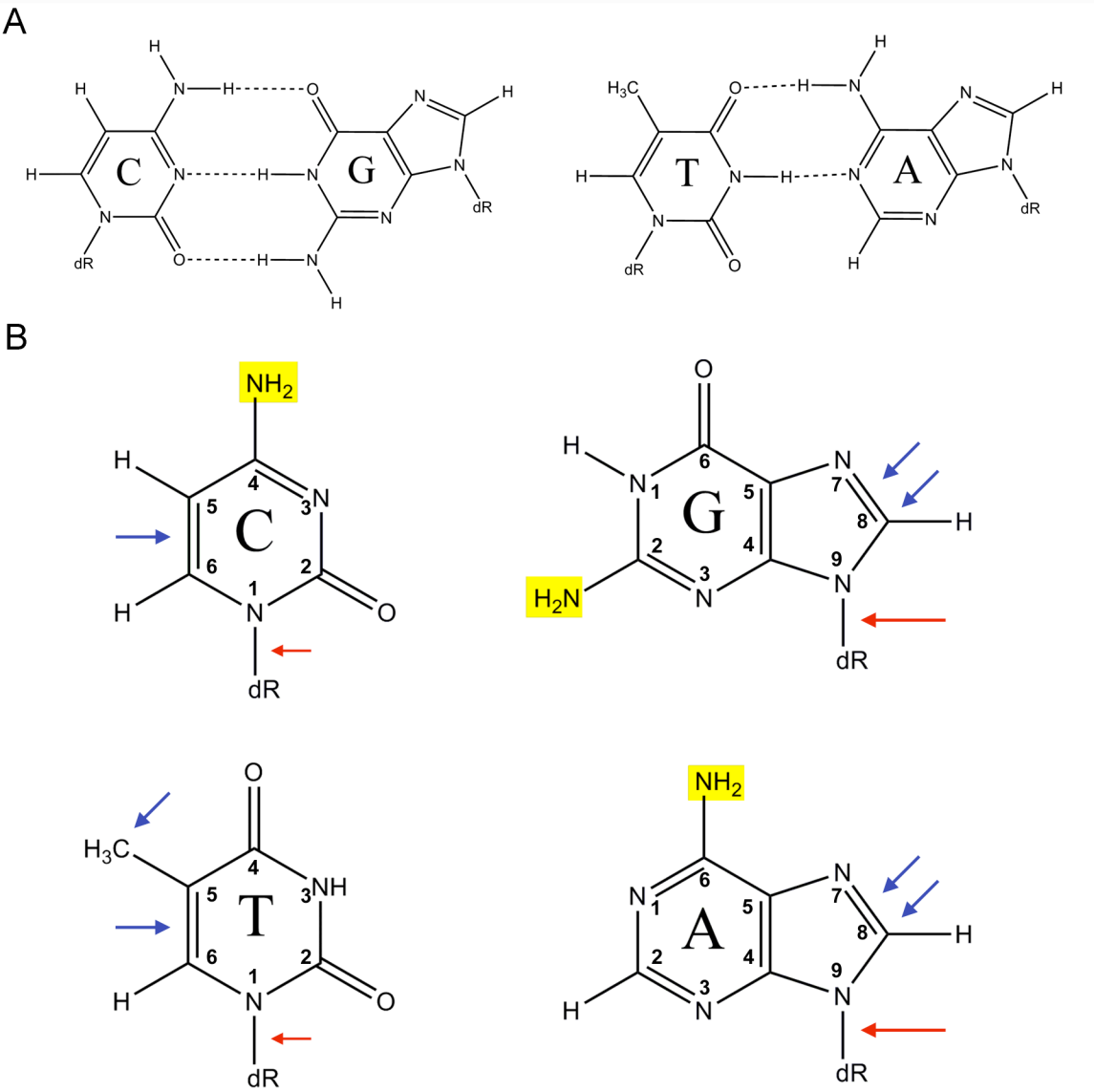

Figure 1: The four principle DNA bases and the major sites of base deamination and hydrolytic and oxidative damage in DNA. A. The four principle DNA bases are shown in their proper base pairs. Dashed lines indicate hydrogen bonds between bases. **B.** The sites of base deamination and hydrolytic and oxidative damage in DNA are shown. The sites of base deamination are indicated by yellow boxes. The major sites of hydrolytic depurination are shown by long red arrows. Short red arrows show other sites of hydrolytic attack. Major sites of oxidative damage are indicated by the blue arrows. dR, deoxyribose; G, guanine; C, cytosine; T, thymine; A, adenine. Modified and reprinted by permission from Macmillan Publishers Ltd: Nature (1), copyright (1993).

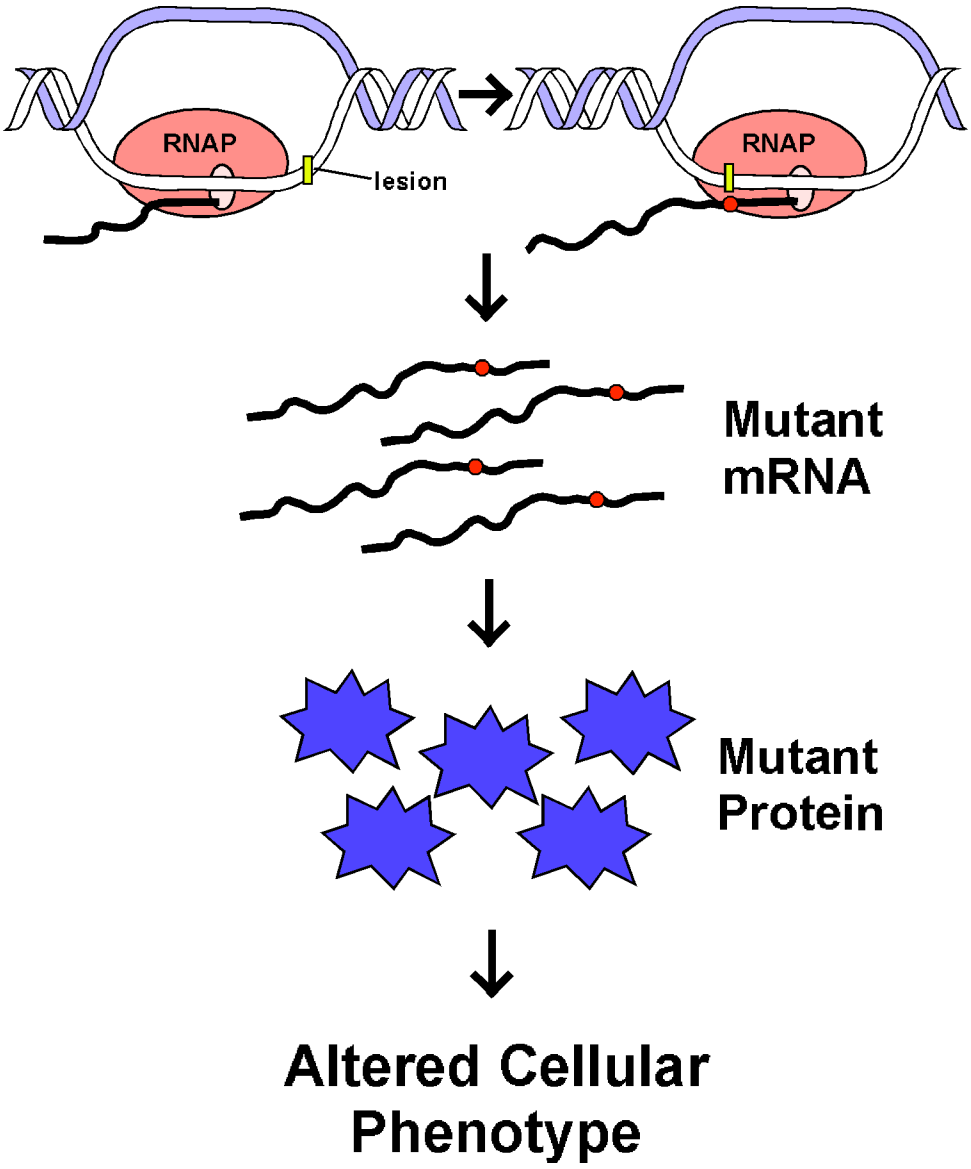

Figure 2: Transcriptional mutagenesis. Transcription past a DNA lesion (yellow box) with altered base pairing properties may lead to the production of a population of mutant transcripts. These transcripts can, in turn, be translated into mutant proteins that could alter the phenotype of the cell. Reproduced with permission from Saxowsky and Doetsch, Chem. Rev. (5) Copyright 2006 American Chemical Society.

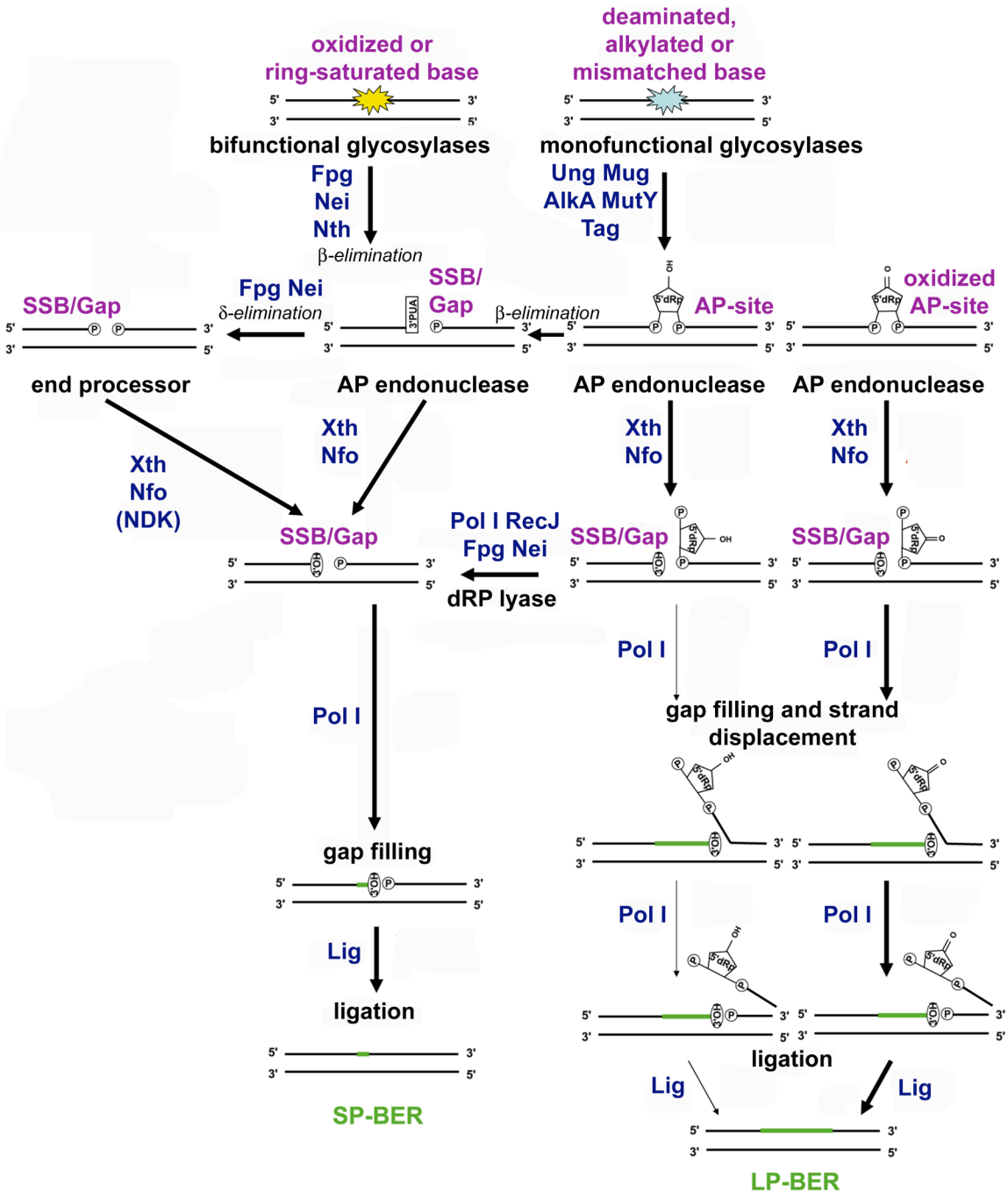

Figure 3: Model for base excision repair in *E. coli*. P, phosphate; OH, hydroxyl group; 3′PUA, 3′-phospho-α,β-unsaturated aldehyde; 5′-dRP, 5′-deoxyribose phosphate. The types of DNA lesions repaired by base excision repair are marked in purple. Enzyme activity is written in black. *E. coli* enzyme names are in blue. Details of end structures in Figure 4. Reprinted and modified with permission from Krwawicz et al., Acta Biochimica Polonica, **54**: 413-434, 2007 (39).

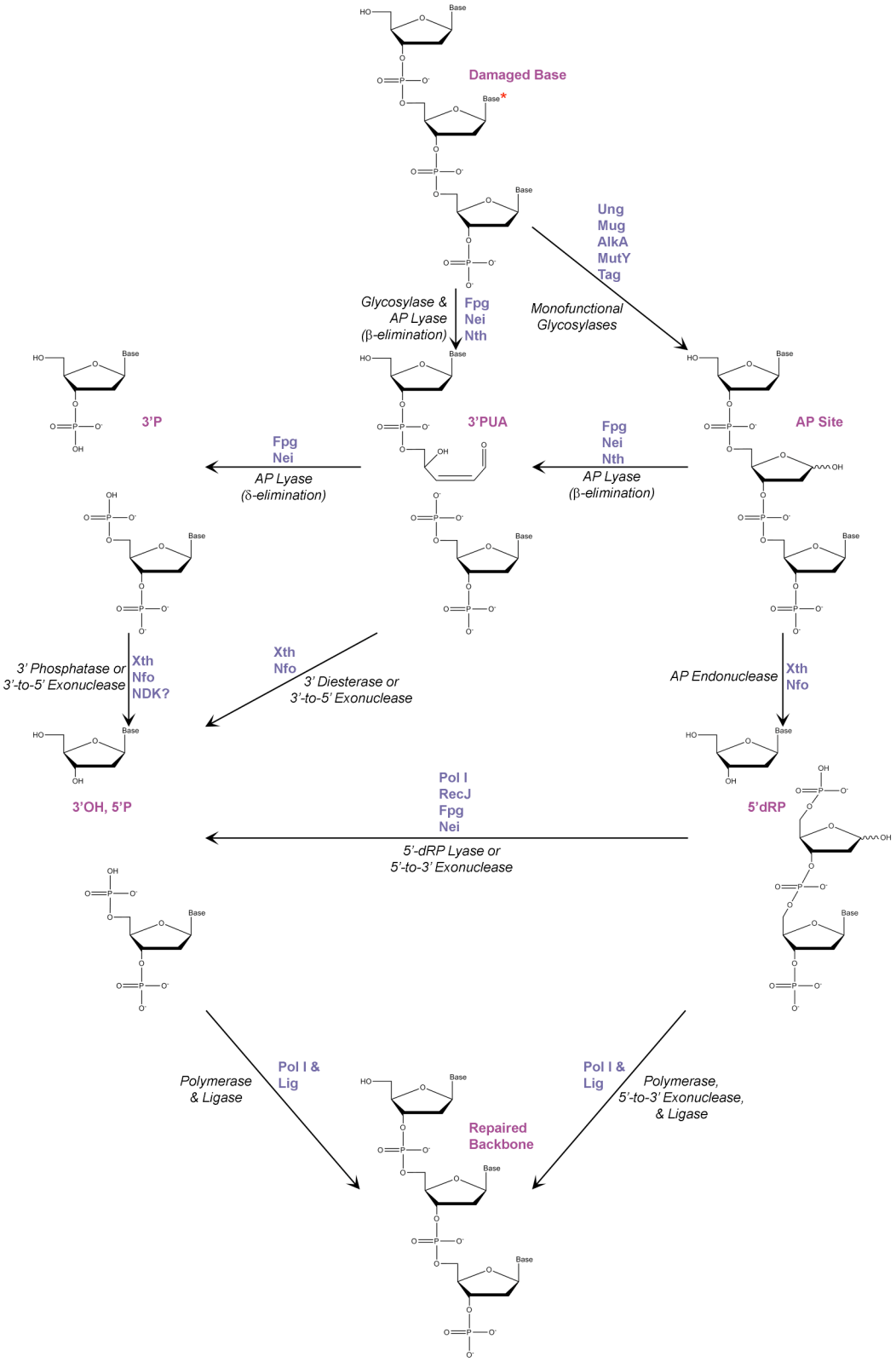

Figure 4: Details of base excision repair end structures in *E. coli*. P, phosphate; OH, hydroxyl group; 3′PUA, 3′-phopho-α,β-unsaturated aldehyde; 5′-dRP, 5′-deoxyribose phosphate. The types of DNA lesions are marked in purple. Enzyme activity is written in black. *E. coli* enzyme names are in blue.

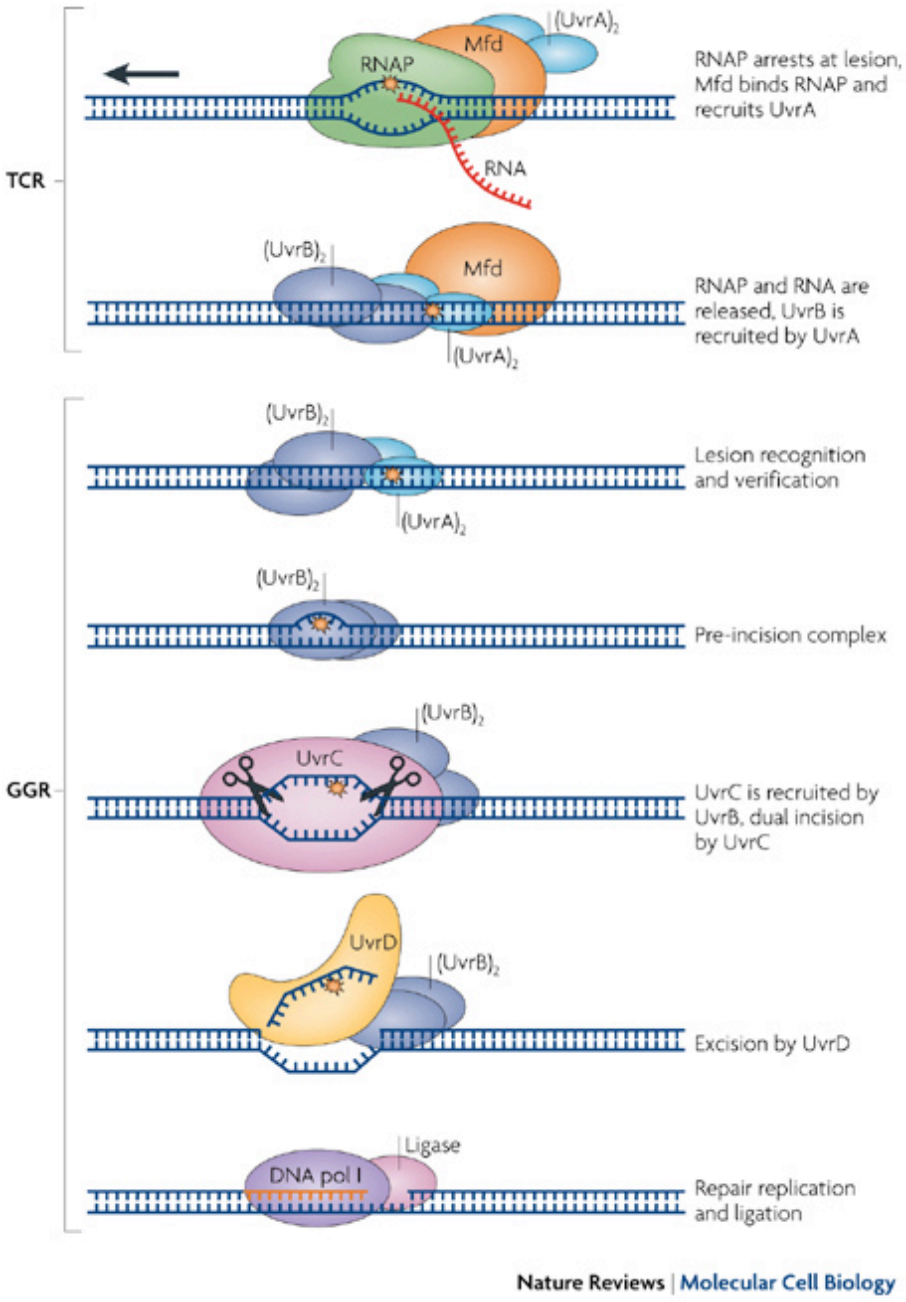

Figure 5: Transcription-coupled and global genome repair in *E. coli*. The arrest of *E. coli* RNA polymerase (RNAP) provides the initial step to highlight a potential lesion: the arrested polymerase recruits Mfd, which then recruits UvrA. Once the UvrA homodimer is bound, it recruits the UvrB helicase, which also operates as a homodimer (61), to verify the presence of a lesion and to determine which strand is damaged. Mfd is released, and the transcription-coupled repair (TCR) reaction proceeds as for global genomic repair (GGR), in which UvrA is the primary element for lesion recognition. The cutting enzyme UvrC is recruited and activated by UvrB to make incisions on each side of the lesion ~12 nucleotides apart. The damaged oligonucleotide is then removed by the UvrD helicase. DNA polymerase-I (DNA pol I) performs repair replication and ligase joins the completed repair patch to the contiguous DNA strand. The entire GGR pathway requires only six proteins and has been reconstituted in vitro (202). Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews (29), copyright (2008).

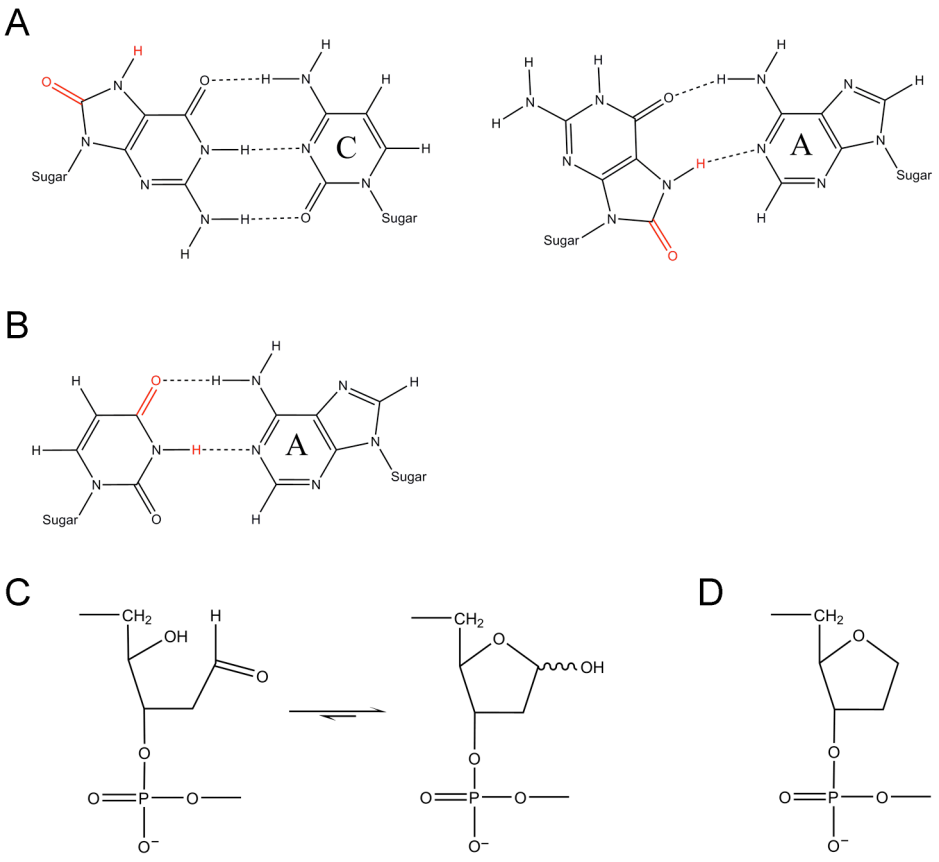

Figure 6: DNA damage examined in these studies. A. Base pairing properties of 8-oxoguanine. Sites of oxidative damage are marked in red. C=cytosine, A=adenine. **B.** Base pairing properties of uracil. Site of deamination marked in red. **C.** Abasic site. Both the 2-deoxy-D-ribose and ring-opened aldehyde forms are shown. **D.** Tetrahydrofuran, abasic site analog.

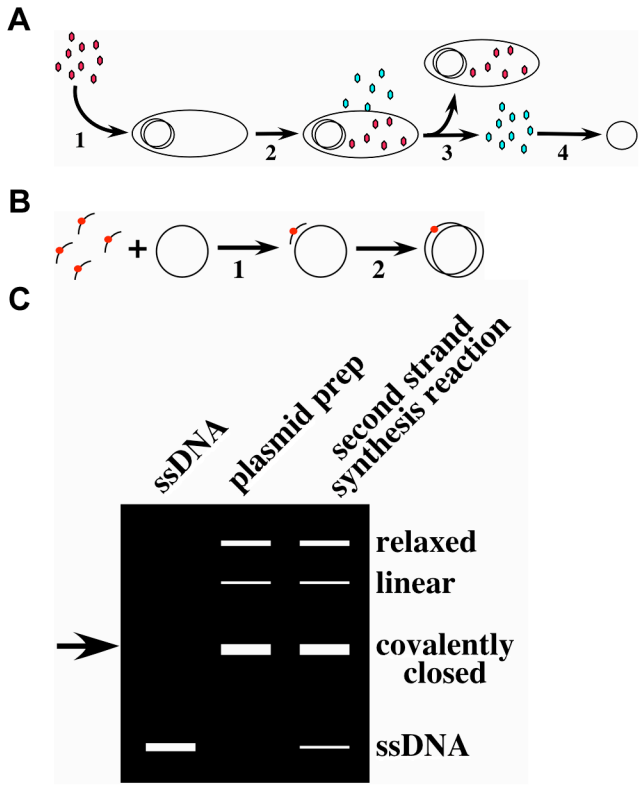

Figure 7. Critical steps for the generation of double-stranded DNA vectors containing site-specific base modifications. A. Bacteria carrying plasmid of choice are infected with M13K07 bacteriophage and cultured overnight. Bacteria are then pelleted by centrifugation and the supernatant containing the phage is collected. Phage are lysed and ssDNA isolated. B. A primer containing the DNA damage of interest is annealed to the ssDNA. The second strand is synthesized in a reaction containing T4 DNA polymerase and T4 DNA ligase. C. The resulting products are resolved on a 0.6% low-melt agarose gel containing ethidium bromide and the covalently closed species is gel purified. Figure courtesy of Tina Saxowsky.

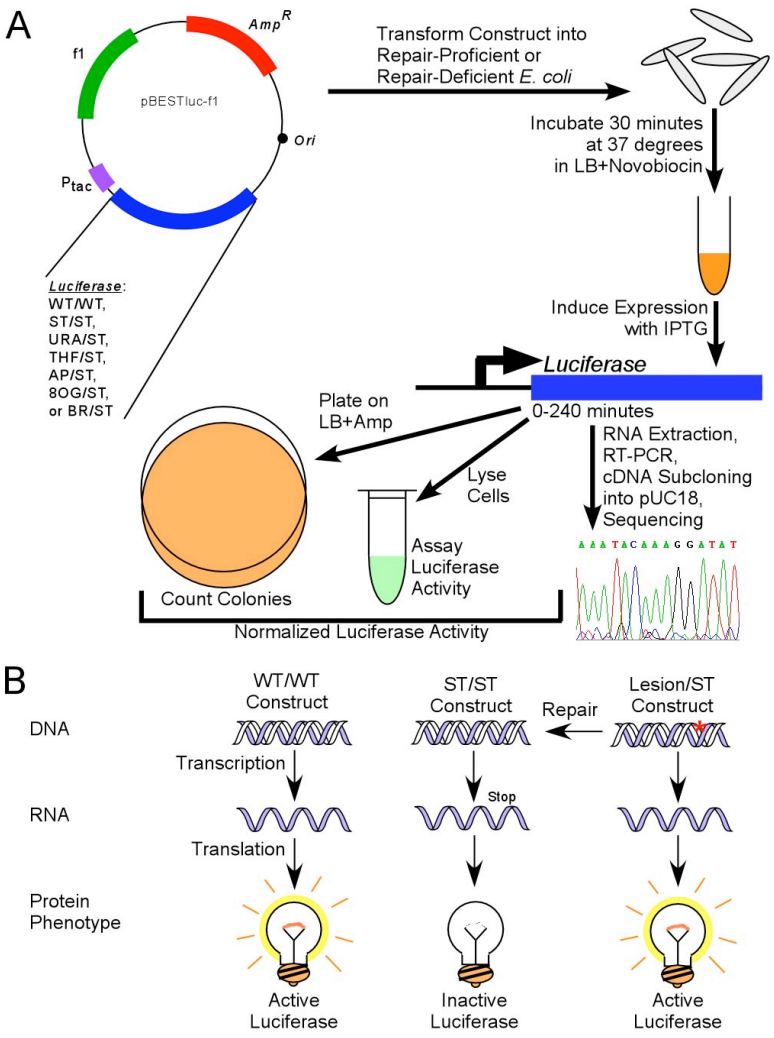

Figure 8. Transcriptional Mutagenesis Luciferase Assay System (TM-LAS)
A. The pBESTluc-f1 luciferase reporter construct contains the firefly luciferase gene (blue) driven by the *E. coli tac* promoter (purple). It also contains the ampicillin resistance gene (red), the f1 origin of replication (green), and an origin of double-strand DNA replication (Ori). At codon 445 of the luciferase gene, sequence is either Normal/Normal (wild-type sequence of codon 445, AAA on coding strand), Stop/Stop (codon 445 changed to Stop codon, TAA on coding strand), or Lesion/Stop (first position of codon 445 in noncoding strand contains a lesion, XTT, coding strand is TAA Stop codon). Constructs are electroporated into repair-proficient and -deficient *E. coli* cells subsequently incubated in LB medium containing novobiocin for 30 minutes. Luciferase gene expression was induced by the addition of IPTG in the medium. At different times following luciferase induction (0-240 minutes), aliquots of cells were lysed and assayed for luciferase activity and also were plated onto LB-Amp medium to normalize luciferase activity. Additionally, at different times following luciferase induction, RNA was isolated and subjected to RT-PCR and subcloning into pUC18 for transcript sequencing. Scheme modified from previously published work (32). B. Predicted results of luciferase assay for luciferase expressed from Normal/Normal, Stop/Stop, Lesion/Stop constructs. Normal/Normal will produce full-length, wild-type luciferase enzyme. Stop/Stop will produce a truncated luciferase enzyme, which will be inactive (201). Lesion/Stop will produce full-length, active luciferase only when RNAP bypasses the lesion and inserts a nucleotide other than uracil opposite the lesion. Once the lesion is repaired, it will be repaired to the Stop codon sequence, producing truncated, inactive luciferase.

Table 1. RNAP Bypass Efficiencies and Insertion Events at Sites of DNA Damage

DNA damage type	RNA polymerase type	Relative bypass efficiency[a]	Nucleotide(s) inserted[a,b]	Outcome	References
abasic site	phage (SP6, T7)	moderate to high	A	transition or transversion	(4, 10, 21)
	E. coli	moderate	A	transition or transversion	(4, 8, 10)
	mammalian RNAP II	variable	C	transition or transversion	(6, 21)
tetrahydrofuran	phage (T7)	high	A, G	transition or transversion	(14)
2-deoxyribonolactone	phage (T3, T7)	none	N.D.		(22)
	mammalian RNAP II	low	N.D.		(22)
uracil	phage (SP6, T7)	high	A	transition	(4, 7, 11)
	E. coli	high	A	transition	(4, 8-10), (32, 33)
	mammalian RNAP II	high	A, G	transition or nonmut.	(6)
dihydrouracil	phage (SP6, T7)	high (pauses)	A, G	transition or nonmut.	(7)
	E. coli	high (pauses)	A, G	transition or nonmut.	(18)
5-hydroxyuracil	mammalian RNAP II	moderate	N.D.		(13)
5-hydroxycytosine	mammalian RNAP II	high	N.D.		(15)
thymine glycol	phage (T7)	moderate	N.D.		(19, 20)
	mammalian RNAP II	moderate to high	A	nonmut.	(13, 15, 17, 19, 20)
8-oxoguanine	phage (T7)	high	A, C	transversion or nonmut.	(14, 16)
	E. coli	moderate to high	A, C, del	transversion, frame shift or nonmut.	(8, 9), (32)
	mammalian RNAP II	moderate to high	A, C, del	transversion, frame shift or nonmut.	(6, 13, 15, 16), (34-36)
8-oxoadenine	mammalian RNAP II	moderate	U > A	nonmut. or transversion	(17)
2-hydroxyadenine	mammalian RNAP II	low	U	nonmut.	(17)
O^6-methyl guanine	phage (T7)	high	U = C >> A	nonmut., transition, transversion	(12)
	E. coli	high	U	transition	(9)
	mammalian RNAP II	moderate	C > U	nonmut., transistion	(12)
5-guanidino-4-nitroimidazole	phage (T7)	high	C > A > del > G >>> U	various	(24)
	mammalian RNAP II	low	C	nonmut.	(24)
AF-guanine	phage (T7)	moderate	C	nonmut.	(14)
	mammalian RNAP II	high (pauses)	N.D.		(25)
AAF-guanine	phage (T7)	low	C	nonmut	(14)
BPDE-adenine (-)	phage (T7)	moderate	A, G, del	transversion or frame shift	(27)
BPDE-adenine (+)	phage (T7)	low	A, G, del	transversion or frame shift	(27)
BPDE-guanine adducts	phage (T7)	low	C	nonmut.	(26, 27)
single strand breaks/gaps	phage (SP6, T7)	variable	del.	frame shift	(11, 30, 31)
	E. coli	variable	del.	frame shift	(8, 30)
	mammalian RNAP II	low	N.D.		(15)
8,5'-cyclo-2'-deoxyadenosine	mammalian RNAP II	moderate	U, del	nonmut. or frame shift	(28)
cyclobutane pyrimidine dimer	mammalian RNAP II	very low	del	frame shift	(28)

[a]References that are underlined represent studies performed *in vivo*. [b]ND = not determined. Table reproduced with permission from Saxowsky and Doetsch, Chem. Rev. (5)

Table 2. Genes involved in BER

Role	Comments	*Escherichia coli* Gene(s)	Human Gene(s)
rMonofunctional DNA N-Glycosylase	Hydrolysis of N-glycosidic bond between a 2′-deoxyribose and the base	*ung* *mug* *alkA* *mutY* *tag*	*UNG* *TDG* *SMUG* *UDG* *MPG* *MUTYH* *MBD4*
Bifunctional DNA N-Glycosylase	Hydrolysis of N-glycosidic bond between a 2′-deoxyribose and the base, and 3′-AP-lyase activity incision of the phosphodiester bond at the 3′ side of the deoxyribose via β- or β,δ-elimination	*fpg* (β,δ) *nei* (β,δ) *nth* (β)	 *OGG* (β) *NEIL1* (β,δ) *NEIL2* (β,δ) *NEIL3* (β) *NTHL1* (β)
AP endonuclease	Cleavage of the phosphodiester bond at the 5′ side of the intact AP-site	*xth* *nfo* *ndk?*	*APEX1* *APEX2*
Removal of 3′-PUA, 3′-P	3′-to-5′ exonuclease, 3′ diesterase or 3′ phosphatase activity	*xth* *nfo* *ndk* (3′-P only)	*APEX1* *APEX2* *PNKP* (3′-P only) *APTX*
Removal of 5′-dRP	5′-dRP-lyase or 5′-to-3′ exonuclease activity	*fpg* *nei* *recJ* *PolA*	 *FEN1* *HMGB1* *POLB* *POLL*
LP-BER flap removal	5′-to-3′ exonuclease activity	*polA*	 *FEN1*
Gap filling	Polymerase activity	*polA*	 *POLB* *POLD* *POLE*
Nick Sealing	Ligase activity	*ligA*	 *LIG1* *LIG3*

AP, apurinic/apyrimidinic (abasic); 3′-PUA, 3′-unsaturated aldehyde; 3′-P, 3′ phosphate; 5′-dRP, 5′-deoxyribose phosphate

Table 3. Genes involved in NER

Human gene*	Role	Comments	*Escherichia coli*
GGR genes			
XPE (also known as *DDB2*)	Lesion recognition	Recruits XPC and is p53 inducible	Unknown
DDB1	Lesion recognition	Forms a complex with DDB2	Unknown
XPC	Lesion recognition	Opens DNA and is p53 inducible	*UvrA*
RAD23B	Lesion recognition	Forms a complex with XPC	Unknown
Centrin-2	Lesion recognition	Forms a complex with XPC	Unknown
GGR and TCR genes			
XPB	Helicase and ATPase	TFIIH subunit[§]	*UvrB*
XPD	Helicase and ATPase	TFIIH subunit[§]	*UvrB*
XPA	Lesion verification	Stabilizes pre-incision complex for GGR and TCR	Unknown
RPA p70, p32 and *p14*	ssDNA binding	Binds to XPA	*Ssb*?
XPF	Structure-specific endonuclease	3′ incision	*UvrC*
ERCC1	Forms a complex with XPF	3′ incision	Unknown
XPG	Structure-specific endonuclease	5′ incision and stablilization of TFIIH	*UvrC*
PCNA	DNA replication sliding clamp	Three subunits; contains docking sites for DNA pol	β-clamp
RFC1	Loads PCNA onto DNA	RFC large subunit	Unknown
Unknown	Removal of incised oligo	None	*UvrD*[‖]
DNA pol δ or ε	DNA replication and repair	None	DNA pol I
DNA pol κ	Bypass polymerase	None	*Dinb1*
DNA ligase-I	Ligase	None	Ligase
DNA ligase-III	Ligase complex	Might be associated with dividing or non-dividing cells	Ligase
XRCC1	Ligase complex	Might be associated with dividing or non-dividing cells	Unknown
TCR genes			
CSA	Ubiquitin-ligase complex	WD repeat	Unknown
CSB	TCR coupling factor and chromatin remodeling	Transcription elongation factor	*Mfd*
XAB2	Transcription factor	Link between XPA and RNAPII	Unknown
TFIIS	RNAPII elongation factor	Stimulation of transcript cleavage by RNAPII	*GreA* and *GreB*
HMGN1	Chromatin relaxation	Nucleosome removal?	Unknown
p300	Chromatin remodeling	Nucleosome removal?	Unknown

*Complexes are indicated by the same adjacent background colors. Other gene products, such as FEN1, MMS19L and p53 might participate in NER or induce expression of NER genes. [‡]Genetic and functional homologues. [§]Although only XPB and XPD have NER functions, all ten TFIIH subunits are essential for NER. [‖]There are no mammalian homologues of UvrD; its role in removing the damaged oligonucleotide might be carried out by DNA polymerases. *ERCC*, excision repair cross-complementing; GGR, global genomic repair; NER, nucleotide excision repair; *PCNA*, proliferating cell nuclear antigen; Pol, polymerase; *Ssb*, single-stranded DNA-binding protein; ssDNA, single-stranded DNA; *RF*, replication factor; RNAP, RNA polymerase; *RPA*, replication protein A; TCR, transcription-coupled repair; TF, transcription factor; XAB2, XPA-binding protein-2; XP, xeroderma pigmentosum. Modified and reprinted by permission from Macmillan Publishers Ltd: Nature Reviews (29), copyright (2008).

CHAPTER 2

DYNAMIC FLEXIBILITY OF DNA REPAIR PATHWAYS IN GROWTH ARRESTED *ESCHERICHIA COLI*

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C.L. Clauson's contribution to this work includes all DNA damage-containing construct preparation, luciferase assays, new strain construction, data analysis and a significant written contribution.

ABSTRACT

The DNA of all organisms is constantly damaged by exogenous and endogenous agents. Base excision repair (BER) is important for the removal of several non-bulky lesions from the DNA, however not much is known about the contributions of other DNA repair pathways to the processing of non-bulky lesions. Here we utilized a luciferase reporter system to assess the contributions of transcription-coupled repair (TCR), BER and nucleotide excision repair (NER) to the repair of two non-bulky lesions, 8-oxoguanine (8OG) and uracil (U), *in vivo* under non-growth conditions. We demonstrate that both TCR and NER are utilized by *Escherichia coli* to repair 8OG and U. Additionally, the relative level of recognition of these lesions by BER and NER suggests that TCR can utilize components of either pathway for lesion removal, depending upon their availability. These findings indicate a dynamic flexibility of DNA repair pathways in the removal of non-bulky DNA lesions in prokaryotes, and reveal their respective contributions to the repair of 8OG and U *in vivo*.

INTRODUCTION

Most cells in nature are not in a constant growth state and are not engaged in continuous rounds of replication (1). Thus, the functional viability of most cell populations likely depends more on the fidelity of transcription and translation than on replication. Maintenance of active regions of the genome is a biological priority as cells have evolved transcription-coupled repair (TCR), a system that preferentially targets repair of bulky DNA damage on the template strand of actively transcribed genes. TCR is mediated in *E. coli* by Mfd, which initiates DNA repair by recognizing a stalled RNA polymerase (RNAP), with subsequent targeting of nucleotide excision repair (NER) components (2).

Previous work from our laboratory demonstrated that non-bulky DNA damage such as 8-oxoguanine (8OG) and uracil (U) can be bypassed by RNAP in a mutagenic manner in *E. coli* leading to transcriptional mutagenesis (TM). Interestingly, it was also observed that the TM caused by 8OG was significantly elevated in the absence of Mfd, indicating that TCR acts on such oxidative DNA base damage *in vivo* (3). Although base excision repair (BER) is the major pathway for transcription-independent repair of 8OG, a synergistic increase in TM in the TCR, BER-defective mutant reveals that Mfd utilizes components outside of BER to facilitate repair. While nucleotide excision repair (NER) proteins are known to interact with Mfd for repair of bulky DNA damage, they are not known to directly act on 8OG (4).

These above studies suggested a dynamic flexibility of DNA repair pathways for the repair of certain types of DNA damage. Such flexibility of pathway utilization could

confer an ability for cells to adapt to changing environments and exposures to mutagens. Mutagens can introduce a spectrum of DNA damage at various locations throughout an organism's genome (5). If the bulk of this DNA damage is handled by a single DNA repair pathway, mutagen exposure could overwhelm single pathways, reducing expression of critical housekeeping genes, leading to cell death. It would be beneficial to the viability of an organism, therefore, if it were able to fall back on secondary overlapping repair pathways to maximize DNA lesions removal. This repair flexibility would restore normal cell function more quickly and reduce the likelihood of cell death due to mutagen exposure. Indeed, it has been observed in the yeast *Saccharomyces cerevisiae* that there is overlap of repair by BER, NER, recombination and translesion synthesis in replicating cells, and that compromising multiple pathways simultaneously is much more detrimental to integrity of the genome than corruption of any pathway individually (6, 7). While these previous studies did not examine which lesions specifically are subject to repair by multiple pathways, they suggested that many oxidative DNA base damages could be accommodated by BER, NER and damage tolerance pathways. In the present study, we addressed whether flexibility of DNA repair pathways can be observed in *E. coli* for two spontaneous, frequently occurring non-bulky lesions, U and 8OG.

Since oxidative DNA base damage (8OG) is subject to TCR (3) in *E. coli*, it was of interest to expand our studies to other non-bulky lesions primarily repaired by BER. To address this issue, we chose to examine whether U was also repaired by TCR *in vivo* under non-growth conditions. Both U and 8OG are known to be mutagenic for both DNA polymerases and RNAPs (5, 8). Additionally, repair by NER of non-bulky DNA

damage was examined both as a possible component of TCR, and as a direct repair mechanism for these lesions. An important goal of this study was to determine whether these non-bulky lesions, which arise by very different mechanisms, are both subject to DNA repair flexibility under non-growth conditions.

MATERIALS AND METHODS

Strain Constructions/Growth Conditions. The genotypes and sources of the *E. coli* strains used in this study are listed in Table 1. Cell growth media was liquid LB supplemented with the following antibiotics, when appropriate: kanamycin (50 μg/ml), tetracycline (15 μg/ml) and ampicillin (50 μg/ml). For newly constructed strains, generalized transduction was performed with phage P1 Δ*dam rev6* as described previously (9) with the donor and recipient designated P1(donor) x recipient in Table 1. Ultraviolet sensitivity of *uvrA* strains was confirmed for several tetracycline (BW1743 donor) or kanamycin (JW4019-2 donor) resistant transductants. *ung* strains were confirmed using λ_{vir} grown on *dut-1 ung-1* strain, BW313 (10, 11).

Generation of Damage-Containing Construct. Constructs were prepared as previously described (3, 12). The four constructs used in this study are designated as Normal/Normal, Stop/Stop, U/Stop and 8OG/Stop. The first word of the construct name refers to the primer used in the DNA polymerization reaction (Table 2), and the second word refers to the plus strand of the plasmids pBESTluc-f1-Normal or pBESTluc-f1-Stop.

TM Luciferase Reporter System (TM-LAS). TM-LAS is illustrated in Figure 1. Competent cells were prepared as described previously (13) using a 1mM, pH 7.0 HEPES first wash. Luciferase assay was carried out as described previously (3, 14).

RESULTS AND DISCUSSION

To investigate the dynamic flexibility of different DNA repair pathways to repair non-bulky DNA lesions in *E. coli*, we utilized DNA damage-tailored plasmid constructs and employed the transcriptional mutagenesis luciferase assay system (TM-LAS) (detailed in Figure 1) as a tool for the measurement of repair. In this system, the configuration of the damage-containing constructs places specific, known base damage across from a Stop codon sequence, enabling a measurement of transcriptional mutagenesis through production of a full-length luciferase protein product only when an unrepaired lesion is bypassed by RNAP (Figure 1B) (3). Therefore, two factors can affect the total amount of active luciferase produced: (i) the rate of repair of the lesion by the available repair proteins in a cell, which will convert the lesion site to a Stop codon during repair synthesis, and (ii) the level of bypass by the RNAP for the lesion, which affects the total amount of full-length RNA produced from the luciferase gene. By comparing a single lesion (8OG or U) between strains with different DNA repair backgrounds, we were able to determine the relative roles of DNA repair enzymes in the removal of this lesion. Thus TM caused by a U or 8OG is a probe for delineating the flexibility of DNA repair pathways.

By utilizing isogenic *E. coli* strains (Table 1) deficient in various components of DNA repair pathways, it is possible to mimic a state in which a DNA repair pathway is overwhelmed, such as would occur following exposure to a mutagen, and investigate the repair of a specific base lesion under these conditions.

Several recent reports have addressed whether certain non-bulky DNA lesions are handled by TCR in bacteria (15), or mammals (16-20). However, most of those studies have been performed *in vitro* (16, 18, 20). Additionally, the few studies utilizing *in vivo* systems employed cells that were not under any growth restriction (15, 17, 19, 20). Unrestricted continuous growth represents a living state rarely experienced by most cell types. It is conceivable that cells alter their priorities for DNA damage handling depending on whether they are dividing or not. These studies were carried out with *E. coli* under growth restriction, through use of the DNA gyrase inhibitor novobiocin. Novobiocin prevents DNA replication, and its use in these experiments provides a system that more closely resembles a natural non-growth state of bacteria (21).

TCR initiates repair of U in vivo. To determine whether TCR acts on non-bulky base lesions in *E. coli*, we assessed U-mediated TM in *mfd* mutants. Unlike what was previously observed for 8OG (3), we did not observe any significant increase in TM in the *mfd* single mutant (Figure 2A, 2C and Table 2). Therefore, we examined TM in a double mutant, *ung mfd*. At 45 and 120 minutes following induction of transcription, a synergistic effect on TM levels ranging from 2- to 4-fold over TM in the *ung* single mutant was observed in the *ung mfd* double mutant (Figure 2A, Table 2). This result confirms a role for TCR in the repair of U *in vivo*. Therefore, despite the fact that both U and 8OG are primarily repaired by BER in replicating and non-replicating cells, these results indicate that TCR is able to participate in the removal of both lesions.

By eliminating the major uracil DNA glycosylase, Ung, U remains in the DNA for a prolonged period of time and abasic sites resulting from Ung- or Mug-mediated removal of U will be produced more slowly (22). If Mfd recognizes abasic sites instead

of U directly, no increase in TM would be observed in *ung mfd* double mutants compared to the *ung* mutant alone because there would be fewer abasic sites to direct recruitment of Mfd, and TM would be unchanged or reduced. However, when loss of Ung is combined with elimination of Mfd, the increase in TM is synergistic, revealing the role of Mfd-mediated TCR of U (Figure 2A and Table 2).

NER directs repair of U *in vivo*. Because of the observations in yeast documenting the overlap of DNA damage repair by BER and NER (6, 7), we wanted to examine the role of NER in the repair of U. NER could be involved in the repair of U through direct recognition of the lesion, or via initiation by TCR (Mfd-mediated), as discussed above. We observed that in strains lacking UvrA, the initiating protein of the NER pathway, following transcription induction, there was a 5- to 10-fold increase in TM at 120 minutes compared to repair-proficient (WT) cells (Figure 2B, 2C, and Table 2). When BER (Ung) and NER (UvrA) are removed simultaneously, considerable variability in the TM levels results (Figure 2B), although the observed trend supports roles for both BER and NER in the repair of U. It is important to note that these data do not represent NER of repair intermediates, such as abasic sites, based on results from our group and others that have indicated that NER is unlikely to be involved in the repair of abasic sites (23, 24).

Because the above results do not allow us to distinguish between a direct role for NER in the repair of U versus TCR-mediated NER, we utilized a *uvrA mfd* double mutant. This mutant displayed a five-fold increase in TM that was consistently elevated compared to that of the *mfd* strain but not the *uvrA* strain (Figure 2C, Table 2). These data indicate that both Mfd and UvrA can prevent U-driven TM *in vivo* reflecting

flexibility of repair of U *in vivo*. Additionally, these data indicate that a subset of UvrA-mediated NER is not associated with Mfd-mediated TCR, and that there is direct repair of U by NER. These results were surprising since NER is suspected to recognize helix distortion caused by bulky DNA damage (25), and U in DNA would not cause significant helix distortion. However, the fact that NER does not have the lesion recognition specificity characteristic of the N-glycosylases of the BER pathway could make NER especially flexible for the repair of many types of lesions, even those that are considered to be helix non-distorting.

NER is involved in the repair of 8OG in vivo. The finding that NER was capable of U repair prompted us to examine whether NER could also repair 8OG. Although it was previously reported that Mfd is important for the repair of 8OG *in vivo* (3), no studies have addressed the potential role of NER directly; we observed that removal of NER results in a variable increase in TM up to 5-fold, depending on the time following initiation of transcription and the specific *uvrA* allele used, indicating that NER is involved in the repair of 8OG *in vivo* (Figure 2D, 2E, and Table 2). To further confirm the role of NER, we utilized a *uvrA mutM* double mutant. When UvrA is removed together with the 8OG DNA glycosylase for BER (MutM), there is a synergistic increase in TM of three to five-fold compared to the TM caused by removing BER alone (Figure 2D, Table 2), indicating that BER and NER may compete for this lesion *in vivo*, and confirming a role for NER in the repair of 8OG. These results would indicate that sharing repair of a lesion among different excision repair pathways is an important strategy for several non-bulky base damages.

TCR of 8OG *in vivo* can utilize both BER and NER. Similar to the situation with U, it was important to consider the relative roles of NER in both direct recognition of 8OG, as well as its role in TCR-mediated repair of this lesion. At various times following induction of luciferase transcription, an increase in TM up to 12-fold over WT was observed in the individual *uvrA* and *mfd* mutant strains (Figure 2D, 2E and Table 2). The *mfd* mutant demonstrated consistently higher TM than the *uvrA* mutants. Additionally, the *uvrA mfd* double mutant showed an almost three-fold increase in TM compared to the isogenic *uvrA* single mutant at 120 minutes (Figure 2E, Table 2). However, there was no significant difference in TM when the double mutant *uvrA mfd* was compared to the *mfd* single mutant (Figure 2E, Table 2).

If repair of 8OG initiated by Mfd completed via the NER pathway, *mfd* would be epistatic to *uvrA*, and there would be no elevation of TM in the *mfd uvrA* double mutant compared to the *uvrA* mutant. However, there is a significant, three-fold increase in TM in *mfd uvrA* compared to *uvrA*, demonstrating that not all repair initiated by Mfd involves UvrA (NER pathway) involvement, thus substantiating a role for BER components in TCR. This result was completely unexpected as TCR proceeding through a BER mechanism has not been demonstrated previously in any organism.

BER may be the preferred pathway coupled to TCR initiated by Mfd due to its opposite base specificity. Because 8OG can pair with adenine as well as with cytosine, many organisms have two separate N-glycosylases for the processing of 8OG. MutM, discussed above, typically removes the 8OG lesion from the DNA when opposite a cytosine residue, but has little activity when 8OG is opposite an adenine residue (26). If MutM were to remove the 8OG when it was paired with adenine during repair synthesis,

DNA polymerase would insert a mutagenic base (T), resulting in a transversion mutation. Therefore, when 8OG is paired with adenine, a separate N-glycosylase, MutY, will remove the adenine, providing the DNA polymerase with an opportunity to insert a non-mutagenic cytosine opposite the 8OG (26). NER lacks such opposite base specificity, so that if NER is utilized to remove 8OG from the DNA instead of BER, it could result in an increase in transversion mutations. This may be the reason that cells prefer to utilize BER components during TCR over NER in the repair of 8OG. Interestingly, there is a synergistic increase in TM for both U and 8OG when TCR and BER are eliminated simultaneously (Figure 2A, Table 2 and (3)), reflecting that these pathways may compete under certain conditions. Based on these observations, we propose that TCR can utilize either NER or BER components for repair of non-bulky base damage, with BER as the predominant repair pathway (Figure 3).

A New Model for DNA Repair in *E. coli*. The dynamic flexibility of DNA repair of non-bulky lesions would provide cells with a greater capacity for repair, especially under conditions where one repair pathway is compromised or the capacity of a single pathway has been exceeded by the level of DNA damage. Such a situation could occur when a cell is exposed to a DNA damaging agent resulting in a variety of different DNA lesions introduced into the genome at different levels simultaneously, a subset of which may only be primarily repaired by a single pathway (e.g. BER). Here, through the use of *E. coli* DNA repair mutants it is possible to mimic a situation where one pathway is overwhelmed by damage, and unavailable for repair of other lesions. By employing TM-LAS to monitor repair, we revealed a role for TCR and NER in the repair of multiple, frequently occurring, non-bulky lesions. These results were unexpected since

these pathways were suspected to only be used for bulky, helix-distorting lesions. In particular, TCR appears to be able to utilize components of either BER or NER, making the repair of damage in actively transcribed genes particularly flexible. As the majority of cells in nature are not actively dividing or replicating their DNA, dynamic flexibility of repair of active regions of the genome becomes particularly important for maintaining cell viability. Though *in vitro* studies of RNAP encountering many non-bulky lesions demonstrates no stalling during bypass (27-29), it is possible that *in vivo* the RNAP stalls infrequently, recruiting Mfd to the damage and preventing further bypass by RNAP until repair can be completed. Therefore, the magnitude of increase in TM observed in the absence of Mfd is relatively modest due to the low frequency of RNAP stalling, not because non-bulky lesions are poor substrates for TCR (Figure 3).

Our results reveal a novel model of transcriptional encounters with non-bulky DNA damage (Figure 3). Unrepaired lesions can be bypassed by RNAP, leading to TM by insertion of an incorrect ribonucleotide opposite the lesion into the mRNA (3). Importantly, a percentage of the time, RNAP stalls at the damage, Mfd is recruited, and TCR is initiated. Mfd removes RNAP from the lesion and then acts as a placeholder, blocking incoming RNAP from bypassing the damage but leaving the lesion accessible to repair by BER. If BER capacity is exceeded, Mfd can recruit NER to complete repair of the lesion, compensating for the reduced capacity of BER. The discovery that NER components can be used for some level of U and 8OG repair is unexpected and reveals a dynamic flexibility of DNA repair pathway interrelationships *in vivo*.

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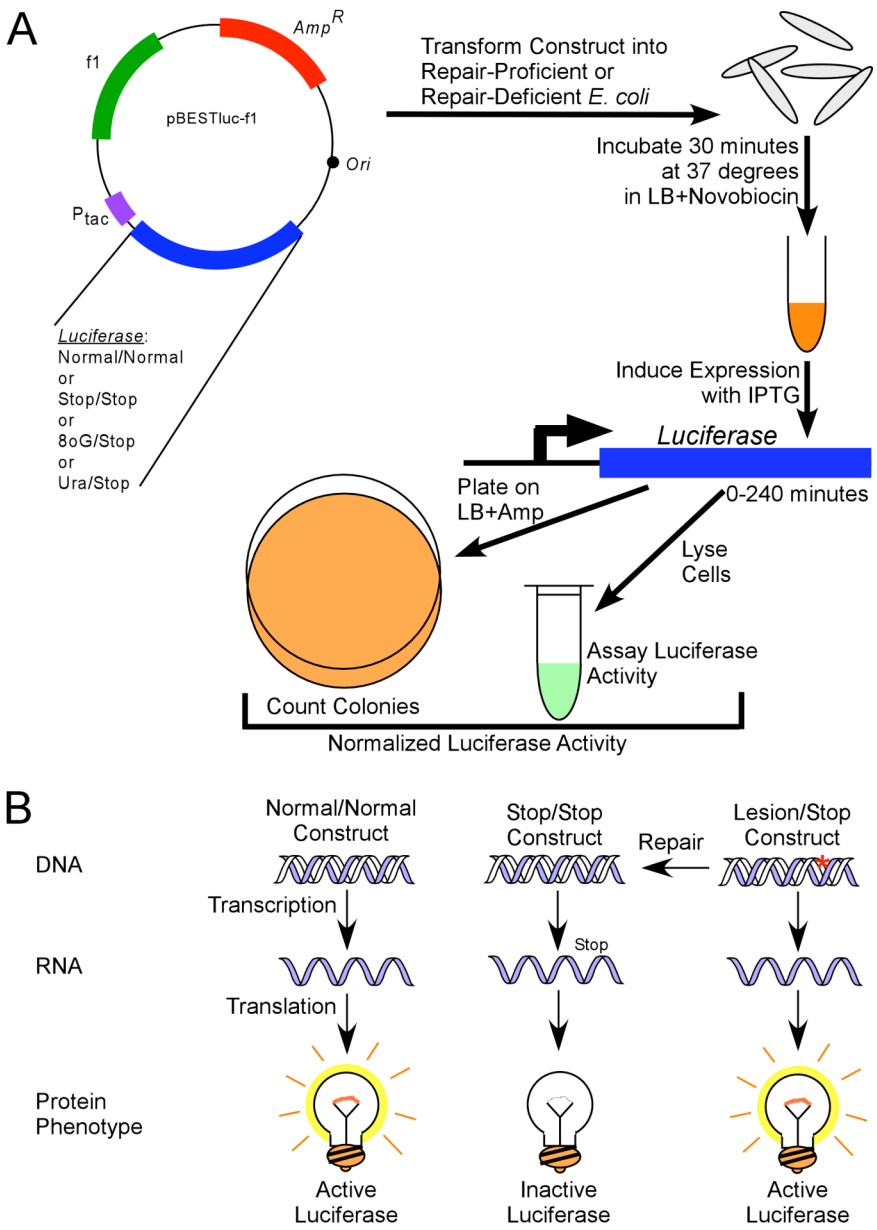

Figure 1. Transcriptional Mutagenesis Luciferase Assay System (TM-LAS)
(A) The pBESTluc-f1 luciferase reporter construct contains the firefly luciferase gene (blue) driven by the *E. coli tac* promoter (purple). It also contains the ampicillin resistance gene (red), the f1 origin of replication (green) for the production of single-stranded DNA corresponding to the coding strand of the luciferase gene, and an origin of double-strand DNA replication (Ori) to allow for its propagation. At codon 445 of the luciferase gene, sequence is either Normal/Normal (wild-type sequence of codon 445, AAA on coding strand), Stop/Stop (codon 445 changed to Stop codon, TAA on coding strand), or Lesion/Stop (first position of codon 445 in noncoding strand contains a lesion, XTT, coding strand is TAA Stop codon). Construct preparation has been described previously (3, 12, 14). Constructs were electroporated into repair-proficient and -deficient *E. coli* cells subsequently incubated in LB medium containing novobiocin for 30 minutes. Luciferase gene expression was induced by the addition of IPTG to the medium. At different times following luciferase induction (0-120 minutes), aliquots of cells were lysed and assayed for luciferase activity and also were plated onto LB-Amp medium to normalize luciferase activity. (B) Predicted results of luciferase assay for luciferase expressed from Normal/Normal, Stop/Stop, Lesion/Stop constructs. Normal/Normal will produce full-length, wild-type luciferase enzyme, resulting in

a high level of luciferase (RLUs). Stop/Stop will produce a truncated luciferase enzyme, which will be inactive (30), and low RLUs. Lesion/Stop will produce full-length, active luciferase only when RNAP bypasses the lesion and inserts a nucleotide other than uracil opposite the lesion. But, once the lesion is repaired, it will be repaired to the Stop codon sequence, producing truncated, inactive luciferase. Thus, a luciferase activity (RLUs) level produced from the Lesion/Stop construct that is greater than the Stop/Stop control construct will indicate RNAP bypass. Figure adapted from Clauson et al., (23).

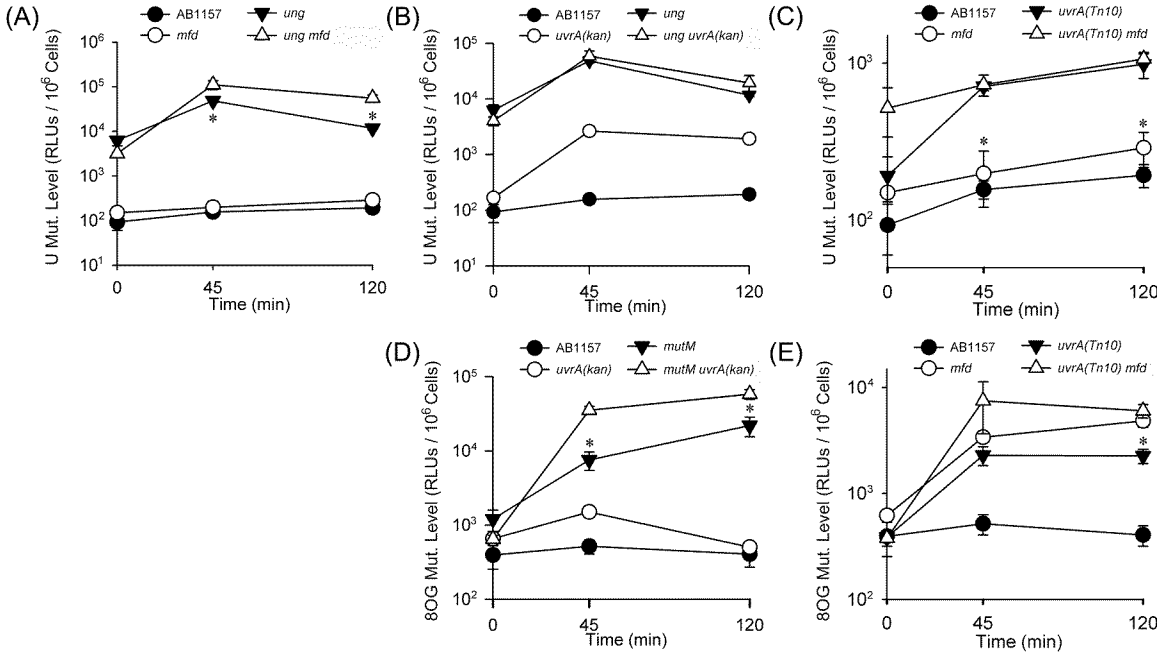

Figure 2: DNA Repair Pathways Involved in Removal of Non-Bulky Lesions from DNA *in vivo*. WT and DNA repair mutant strains (Table 2) were transformed with damage-containing construct, and the level of mutagenesis (measured via luciferase activity and expressed as relative light units per 10^6 cells) was measured at 0, 45, and 120 minutes following IPTG induction using TM-LAS methodology (Supplementary Fig. 1). Each data point represents the mean of at least three replicates ± SEM. Statistical significance was calculated using a Student's t-test. Distributions were considered to be significantly different when $p < 0.05$. (A) U/Stop construct. * Denotes points at which the single mutant, *ung*, is significantly different from the double mutant *ung mfd*. All points for *ung* and *ung mfd* are significantly different from the wild type and *mfd* strains. (B) U/Stop construct. All points for *ung* and *ung uvrA(kan)* are significantly different from the wild type strain. At t=45 and 120 minutes *uvrA(kan)* is significantly different from the wild type strain. (C) U/Stop construct. * Denotes points at which the single mutant, *mfd*, is significantly different from the double mutant *uvrA(Tn10) mfd*. All points for *uvrA(Tn10)* and *uvrA(Tn10) mfd* are significantly different from the wild type strain, but do not significantly differ from each other at any point. (D) 8OG/Stop construct. *Denotes points at which the single mutant, *mutM* is significantly different from the double mutant *mutM uvrA(kan)*. All points after t=0 for *mutM* and *mutM uvrA(kan)* are significantly different from the wild type strain. At t=45 the *uvrA(kan)* strain is significantly different from the wild type strain. (E) 8OG/Stop construct. * Denotes points at which the single mutant, *uvrA(Tn10)*, is significantly different from the double mutant *uvrA(Tn10) mfd*. All points after t=0 minutes for *uvrA(Tn10)*, *mfd*, and *uvrA(Tn10) mfd* are significantly different from the wild type strain.

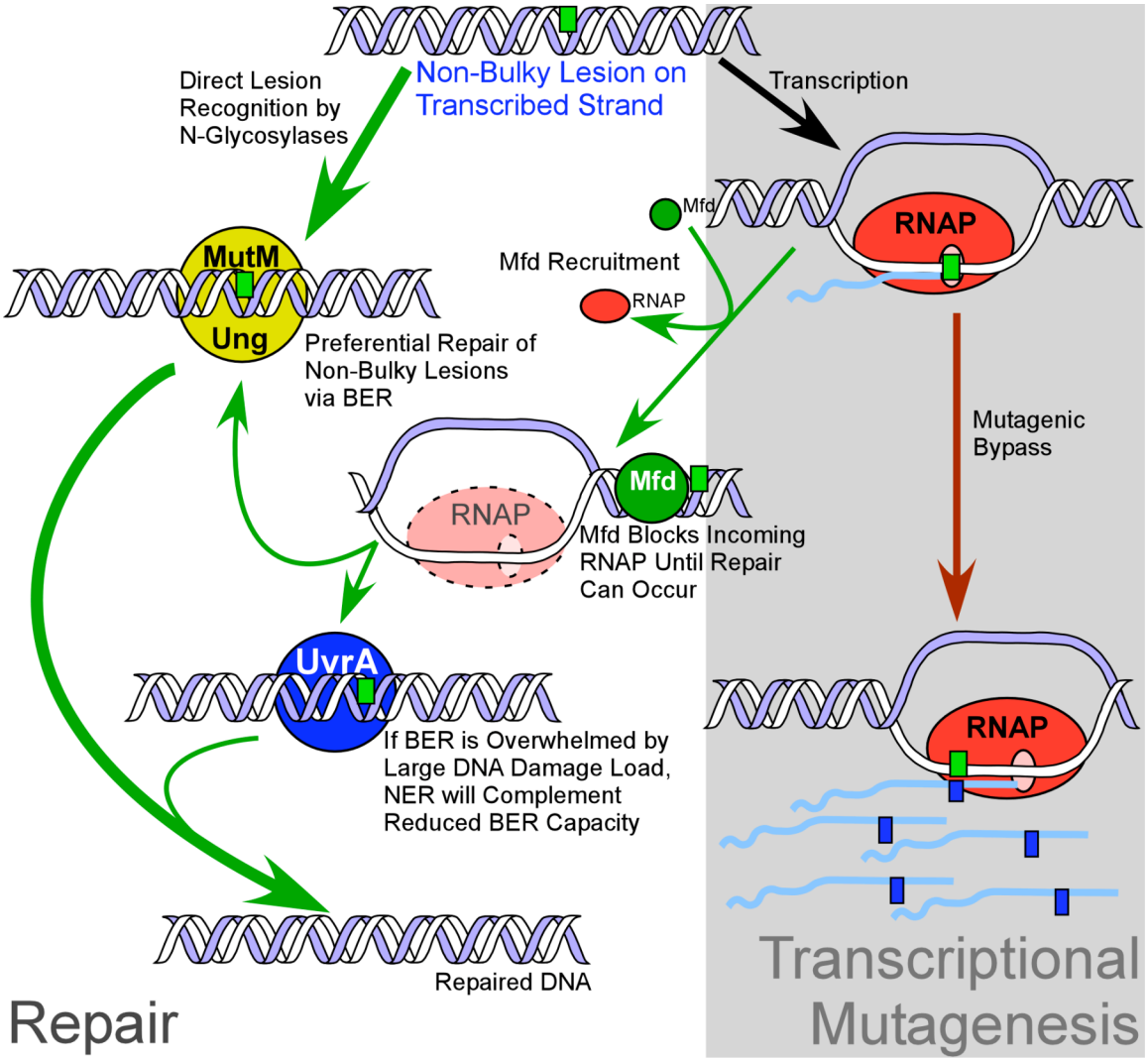

Figure 3: Dynamic Flexibility of Interacting DNA Repair Pathways in *E. coli*. Non-bulky lesions in DNA (small green box) are primarily repaired by the BER pathway. When these lesions are present on the template strand of a gene and are encountered by RNAP, at least two possible outcomes can result: 1. Transcriptional mutagenesis via insertion of an incorrect ribonucleotide opposite the lesion on the nascent mRNA (red arrow), or 2. Mfd-mediated TCR of the lesion (green arrows). Mfd removes RNAP from the lesion and subsequently acts to block incoming RNAP from bypassing the lesion in a mutagenic manner. Mfd leaves the damaged base accessible to BER. However, if the capacity of BER is overwhelmed by excessive DNA damage elsewhere, Mfd can recruit NER to repair the lesion, thus complementing the reduced BER capacity for the repair of small lesions.

Table 1. Strains and Primers

Strains	Description	Source
AB1157	*thr-1*, *araC14*, *leuB6* (Am), Δ*(gpt-pro) lacY1*, *tsx-33*, *qsr'-0*, *glnV44* (AS), *galK2* (Oc), *Rac-0*, *hisG4* (Oc), *rfbD1*, *mgl-51*, *rpoS396* (Am), *rpsL31*, *kdgK51*, *xylA5*, *myl-1*, *argE3* (Oc), *thi-1*	B. Weiss (31)
BD2008	*ung-151*::Tn*10*	(32)
BW313	*dut-1, ung-1*	B. Weiss
BW1743	AB1157 *uvrA::Tn10*	B. Weiss
DH12S	*φ*80d*lacZM*15, *mcrA*, Δ(*mrr-hsdRMS-mcrBC*), *araD*139, Δ(*ara*, *leu*)7697, Δ*lacX*74, *galU*, *galK*, *rpsL*, *deoR*, *nupG*, *recA*1 [F′*pro*AB^{+} *lacI*^{q}ZΔ*M*15]	P. Doetsch collection
JW4019-2	*F-, Δ(araD-araB)567, ΔlacZ4787(::rrnB-3), LAM-, rph-1, Δ(rhaD-rhaB)568, ΔuvrA753::kan, hsdR514*	Coli Genetic Stock Center, Yale University (33)
Z078	AB1157 *mutM::mini- Tn10*	(3)
Z079	AB1157 *mfd98*::Tn*5*	(3)
Z102	AB1157 *mfd98*::Tn*5, uvrA::Tn10*	P1(BW1743) x BW1603
Z105	AB1157 *ung-151::Tn10*	P1(BD2008) x AB1157
Z106	AB1157 *ung-151::Tn10, mfd98::Tn5*	P1(BD2008) x BW1603
Z109	AB1157 *ΔuvrA753::kan*	P1(JW4019-2) x AB1157
Z110	AB1157 *ΔuvrA753::kan, mutM::mini- Tn10*	P1(JW4019-2) x Z078
Z111	AB1157 *ΔuvrA753::kan, ung-151::Tn10*	P1(JW4019-2) x Z105
Primers	Sequence	
Const-Normal	5′phos-CGATTCCAATTAAGCGGGGGCCACCTGATATCCTTTGTATTTAAT-3′	
Const-Stop	5′phos-CGATTCCAATTAAGCGGGGGCCACCTGATATCCTTAGTATTTAAT-3′	
Const-Ura	5′phos-CGATTCCAATTAAGCGGGGGCCACCTGATATCCTTUGTATTTAAT-3′	
Const-8oG	5′phos-CGATTCCAATTAAGCGGGGGCCACCTGATATCCTT*GGTATTTAAT-3′	

Table 2. Luciferase Activities 2 hr Following Induction in 8OG and U Repair-Proficient and Deficient Cells

Strain[a]	RLU[b]/10^6 cells ± SEM[c]	Mutant/Wild-Type[d]	p-Value[e]
Normal/Normal	NTS[f] 5′ TAC AAA GGA 3′ TS[g] 3′ ATG TTT CCT 5′		
AB1157	229000 ± 156000[h]	1.00	
AB1157 *ung*	124000 ± 15300	0.54	0.749
AB1157 *mutM*	111000 ± 7750	0.48	0.604
AB1157 *mfd*	134000 ± 30000	0.59	0.574
AB1157 *uvrA(Tn10)*	218000 ± 45800[h]	0.95	0.948
AB1157 *uvrA(Tn10) mfd*	245000 ± 5080	1.07	0.962
AB1157 *ung mfd*	76800 ± 16000	0.34	0.643
AB1157 *uvrA(kan)*	8670 ± 1860	0.04	0.504
AB1157 *uvrA(kan) mutM*	75900 ± 16300	0.33	0.641
AB1157 *uvrA(kan) ung*	16000 ± 9230	0.07	0.518
Stop/Stop	NTS[f] 5′ TAC TAA GGA 3′ TS[g] 3′ ATG ATT CCT 5′		
AB1157	120 ± 39[h]	1.00	
AB1157 *ung*	342 ± 138	2.86	0.329
AB1157 *mutM*	112 ± 58	0.94	0.881
AB1157 *mfd*	306 ± 76	2.56	0.115
AB1157 *uvrA(Tn10)*	213 ± 73[h]	1.78	0.286
AB1157 *uvrA(Tn10) mfd*	110 ± 31	0.92	0.842
AB1157 *ung mfd*	252 ± 153	2.11	0.420
AB1157 *uvrA(kan)*	32 ± 5	0.27	0.168
AB1157 *uvrA(kan) mutM*	126 ± 95	1.05	0.944
AB1157 *uvrA(kan) ung*	134 ± 36	1.12	0.829
Ura/Stop	NTS[f] 5′ TAC TAA GGA 3′ TS[g] 3′ ATG **U**TT CCT 5′		
AB1157	193 ± 32[h]	1.00	
AB1157 *ung*	11700 ± 711	60.7	<0.001
AB1157 *mfd*	289 ± 73	1.50	0.177
AB1157 *uvrA(Tn10)*	983 ± 90	5.09	<0.001
AB1157 *uvrA(Tn10) mfd*	1060 ± 83	5.50	<0.001
AB1157 *ung mfd*	55500 ± 10200	287	<0.001
AB1157 *uvrA(kan)*	1930 ± 296	9.98	<0.001
AB1157 *uvrA(kan) ung*	19400 ± 6780	101	0.006
8OG/Stop	NTS[f] 5′ TAC TAA GGA 3′ TS[g] 3′ ATG **8**TT CCT 5′		
AB1157	406 ± 89[h]	1.00	
AB1157 *mutM*	19800 ± 7650	48.8	0.003
AB1157 *mfd*	4850 ± 644	11.9	<0.001
AB1157 *uvrA(Tn10)*	2270 ± 350	5.59	<0.001
AB1157 *uvrA(Tn10) mfd*	6040 ± 874	14.9	<0.001
AB1157 *uvrA(kan)*	503 ± 78	1.24	0.4202
AB1157 *uvrA(kan) mutM*	58000 ± 8620	143	<0.001

[a]Full strain descriptions in Table 1.
[b]Relative light units.
[c]Each value is the average of at least three replicate samples ± standard error of the mean.
[d]Ratio of mutant luciferase activity over repair-proficient cells luciferase activity for the same construct.
[e]p-values for Student's t-test comparison between mutant and repair-proficient cells luciferase activity for the same construct. Distributions were considered to be significantly different when $p < 0.05$.
[f]Nontranscribed strand
[g]Transcribed strand; U=uracil, 8=8-oxoguanine
[h]This data included in Clauson et al., (23)

CHAPTER 3

ABASIC SITES AND STRAND BREAKS IN DNA CAUSE TRANSCRIPTIONAL MUTAGENESIS IN *ESCHERICHIA COLI*

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C.L. Clauson's contribution to this work included all DNA damage-containing construct preparation, luciferase assays, new strain construction, *nfo* induction β-galactosidase assay, RNA isolation and cDNA subcloning for transcript sequencing, data analysis and a significant written contribution. K.J. Oestreich and J.W. Austin contributed RT-PCR for transcript sequencing protocol.

ABSTRACT

DNA damage occurs continuously, however faithful replication and transcription are essential for maintaining cell viability. Cells in nature are not dividing and replicating DNA often, and therefore, it is important to consider the outcome of RNA polymerase (RNAP) encounters with DNA damage. Base damage in the DNA can affect transcriptional fidelity, leading to production of mutant mRNA and protein in a process termed transcriptional mutagenesis (TM). Abasic (AP) sites and strand breaks are frequently occurring, spontaneous damages that are also base excision repair (BER) intermediates. In vitro studies demonstrated that these lesions can be bypassed by RNAP, however this has never been assessed in vivo. This study demonstrates that RNAP is capable of bypassing AP sites and strand breaks in Escherichia coli and results in TM through adenine incorporation in nascent mRNA. Elimination of the enzymes that process these lesions further increases TM, however such mutants can still complete repair by other downstream pathways. These results demonstrate that AP sites and strand breaks can result in mutagenic RNAP bypass, and have important implications for the biologic endpoints of DNA damage.

INTRODUCTION

Most cells in nature are in a state of limited growth and are therefore not often engaged in replication (1, 2). Thus, the functional viability of most types of cells likely depends more on faithful transcription and translation than on faithful replication. Several base damages are bypassed in vivo by RNA polymerase (RNAP) in a mutagenic manner, resulting in transcriptional mutagenesis (TM) (3-7). In nondividing cell populations, TM could have a large contribution to the mutant protein burden and resulting cellular phenotype, compared with replication-based mutagenesis events.

Base excision repair (BER) is responsible for the processing of many small lesions, and in particular, those that do not cause significant helix distortion. During BER these lesions are removed by specialized DNA N-glycosylases. Monofunctional glycosylases remove the damaged base, leaving an abasic (AP) site. Bifunctional glycosylase/AP lyase enzymes remove the lesion and nick the DNA strand via β- or β,δ-elimination, resulting in a 3′ blocking group (8). Next, AP endonucleases process the AP sites and 3′ blocking groups to produce 3′-termini compatible with subsequent DNA repair synthesis (8, 9). In vitro, RNAP can bypass several types of repair intermediates, including AP sites and various strand breaks (10-14). Whether RNAP is capable of bypassing abasic sites and strand breaks in live organisms is an important issue for defining the spectrum of DNA damages that can cause TM, as well as for understanding the relationships between RNAP and DNA repair processes. Here we have demonstrated that RNAP is capable of bypassing AP sites and strand breaks in *Escherichia coli*.

MATERIALS AND METHODS

Strains and Media Conditions. The genotype and sources of E. coli strains used in this study are listed in Table 1. New strains were constructed using generalized transduction with phage P1 Δdam rev6, as described previously (15), with the donor and recipient designated "P1(donor) x recipient." UV sensitivity of uvrA strain was confirmed for several tetracycline-resistant transductants. The nfi strain was confirmed for chloramphenicol-resistant transductants using PCR and primers Nfi1 and Nfi2 (Table 1) as previously described (16). Cell growth media was liquid LB supplemented with the following antibiotics, when appropriate: kanamycin (50 μg/mL), tetracycline (15 μg/mL), and ampicillin (50 μg/mL). Competent cell preparation was as described previously (3).

Preparation of Constructs. Preparation of control constructs and constructs containing 8OG, URA, and APTHF was described previously (3). SSB/ST construct was prepared by treating 8OG/ST construct with commercially available Fpg (New England Biolabs) as per the manufacturer´s instructions. The quantitative conversion to a construct containing a SSB/ST was confirmed by agarose gel electrophoresis in the presence of ethidium bromide, wherein the amounts of covalently closed and nicked constructs can be distinguished and assessed. AP/ST construct was prepared by treating URA/ST construct with commercially available UDG (New England Biolabs) as per the manufacturer´s instructions. Presence of the AP site is confirmed through the digestion of the AP construct with Nfo (a generous gift from Yoke Wah Kow, Emory University) and analysis with agarose gel electrophoresis in the presence of ethidium bromide. In

vitro digested constructs were prepared on the day of transformation and filtered on Millipore membrane filters before electroporation.

Transcriptional Mutagenesis Luciferase Assay System (TM-LAS). Luciferase assays were performed as described previously (3). The assay methodology is illustrated in Figure 1.

β-Galactosidase assay for detection of *nfo* induction. β-Galactosidase activity was measured in suspensions of cells grown in the presence or absence of novobiocin and were treated with $CHCl_3$ and SDS (17).

RNA Preparation and RT-PCR. AB1157 *xth nfo nfi* cells were transformed with construct as previously described (3) and grown at 25°C; then RNA was isolated from the bacteria at appropriate time points after IPTG induction. RNA was prepared using PerfectPure RNA Cultured Cell Kit (5Prime), subjected to a two hour DNase I digestion (Baseline-ZERO, Epicentre), and further processed using a DNA-Free RNA Kit (Zymo Research). An additional DNaseI digestion (Promega) containing all components of the subsequent RT-PCR (except random hexamers and reverse transcriptase) was performed for six hours. Approximately 500 ng RNA was reverse transcribed using random hexamers (Applied Biosystems) and then PCR amplified using the primers LBRT1, LBRT2 (Table 1) to prime. Removal of contaminating DNA was confirmed by Taq PCR without a preceding reverse transcriptase step.

Transcript Sequencing. Subcloning of cDNA was carried out by ligating the Sau3AI/HincII fragment of the RT-PCR product between BamHI and HincII sites of pUC18 as previously described (3), using X-Gal and IPTG onLB-Amp plates for blue-white screening. White ampicillin-resistant colonies were subjected to PCR

amplification using Clo18U and Clo18L (Table 1). Sequencing was carried out by Macrogen or Agencourt using Clo18U to prime sequencing reactions.

RESULTS

We investigated TM mediated by AP sites and strand breaks by using damage-tailored plasmid constructs and using a transcriptional mutagenesis luciferase assay system (TM-LAS) (3, 15) (Fig. 1) in isogenic *E. coli* strains with different DNA repair backgrounds (Table 1). In this system, the configuration of the damage-containing constructs places the lesion across from a Stop codon sequence, enabling a measurement of TM through production of a full-length luciferase protein product only when an unrepaired lesion is bypassed by RNAP (Fig. 1) (3). Therefore, two factors can affect the total amount of active luciferase produced: (i) the rate of repair of the lesion by the available repair proteins in a cell, which will convert the lesion site to a Stop codon during repair synthesis, and (ii) the level of bypass by the RNAP for the lesion, which affects the total amount of full-length RNA produced from the luciferase gene. In addition to examining the functional protein product of the luciferase gene, cDNA sequencing was done to determine which nucleotide is inserted opposite the lesion during bypass, making this system particularly useful for examining TM resulting from a variety of DNA damages in vivo.

All experiments were carried out in the presence of the DNA gyrase inhibitor novobiocin to prevent DNA replication and hold the cells in a nongrowth state while allowing transcription to occur (7). By using cells under nongrowth conditions, we were able to study TM and DNA repair in a more natural state (18).

We used three AP site constructs: (i) URA/ST, containing a uracil to be converted to an AP site in vivo; (ii) AP^{THF}/ST, containing the AP site analog tetrahydrofuran that

cannot be processed by AP lyases (17); and (iii) AP/ST, which contains a bona fide AP site created in vitro (Materials and Methods). The

strand break that we investigated is that which is created through the β,δ-elimination activity of AP lyases, containing a one nucleotide gap with phosphate groups on both the 3′ and 5′ ends. We used two strand break constructs: (i) 8OG/ST, which harbors an 8-oxoguanine to be converted to a strand break in vivo; and (ii) SSB/ST, which contains a strand break created in vitro (Materials and Methods).

AP Sites Cause TM in E. coli Deficient in AP Endonuclease Activity. To examine TM caused by AP sites, we transformed the constructs into repair deficient *E. coli*. By comparing a single lesion (AP site) between strains with different DNA repair backgrounds, we were able to determine the relative roles of DNA repair enzymes in the removal of this lesion. First, we examined strains deficient in a single AP endonuclease, either Nfo or Xth, and determined the level of TM. In the *nfo*, but not the *xth* strain, there was a significant increase in TM at 120 min following transformation (Table 2), demonstrating that Nfo, but not Xth, is a major component for prevention of TM caused by AP sites. Although *nfo* is an inducible gene whereas *xth* is not, we were able to show using a β-galactosidase assay (17) with a strain containing an *nfo'-lacZ* fusion (Table 1) that nfo is not induced by treatment with novobiocin (Miller units for media with novobiocin, 766; media without novobiocin, 747). These results indicate that under conditions where nfo is induced, the effect on TM could be greatly enhanced. The removal of both E. coli AP endonucleases (*xth nfo* mutant) resulted in a significant elevation in TM at all timepoints examined (Table 2 and Figure 2A), confirming that TM

is further elevated when repair is substantially compromised. These results indicate that mutagenic RNAP bypass of AP sites occurs in vivo.

There is not a substantial increase in TM following IPTG induction, however, it is known that the promoter used in these studies is leaky, allowing some degree of transcription to occur before IPTG is introduced to the culture media. This leakiness is particularly evident for the Norm/Norm control construct (Figure 3A), where a large amount of luciferase activity is measured at t = 0. Therefore, it is likely that the majority of the TM caused by AP sites is occurring during the 30 minute recovery time, but the lesions are also repaired during this time. This would mean that an increase in transcription at t = 0 would be an increase of transcription of repaired constructs, that would not yield active luciferase, and, therefore, the total amount of active luciferase would remain unchanged by IPTG induction. Although it might appear that at t = 0 the cells transformed with URA/ST construct displayed a lower level of TM compared with cells transformed with AP/ST or AP^{THF}/ST, these differences were not statistically significant (Figure 2A).

Interestingly, despite in vitro evidence that an alternative repair enzyme, Nfi, can recognize AP sites (16, 19, 20), here we observed a suppressive effect on the levels of TM caused by AP sites when Nfi was deleted (Tables 2 and 3). Although we currently do not have an explanation for the observed suppressive effect, the same pattern is observed in the several different repair backgrounds examined.

RNAP Bypass of AP Sites Results in Production of In-Frame Mutant Transcripts. Next, we determined the bases inserted by RNAP opposite the AP site. By using an *xth nfo nfi* strain we found that, for AP sites and AP^{THF}, RNAP inserted

exclusively adenine with no deletion events observed (Figure 2B). An AP^{THF}/WT construct was used to distinguish between uracil that is incorporated opposite a repaired lesion or that which results from RNAP insertion of both adenine and uracil opposite AP sites. In the AP^{THF}/WT construct, repair of the AP^{THF} would lead to thymine on the template strand during repair synthesis, subsequently coding for adenine during transcription. As we observed only adenine incorporation, we conclude that RNAP preferentially inserts adenine opposite AP sites, and all uracil incorporation observed for the Lesion/ST constructs results from transcription events across repaired templates.

cDNA sequencing not only reflected RNAP insertion events but also revealed rates of DNA repair and provided information about the DNA repair pathways involved in the repair of AP sites. It is difficult to infer rates of conversion of uracil to an AP site in vivo, as the sequencing results of transcription past the URA/ST construct revealed an unusual pattern of repair, which we cannot explain at this time. However, when we compare AP/ST and AP^{THF}/ST, we observe that AP^{THF} was repaired more slowly than AP (Figure 2B). Because AP^{THF} cannot be processed by AP lyases, this indicates a role for AP lyases in the repair of AP sites in vivo (21). In addition, the use of the triple mutant *xth nfo nfi* strain for cDNA sequencing allowed us to simultaneously exclude three important DNA repair enzymes that could be involved in the repair of AP sites. It is interesting, therefore, that the transcript sequencing results demonstrate that repair of AP^{THF} occurs when AP endonuclease and Nfi-mediated repair is compromised under conditions when AP lyase cleavage can be excluded as a repair mechanism. Also, eliminating a nucleotide excision repair (NER) protein, UvrA, did not have any effect on TM caused by AP sites (Table 3). As indicated in the previous section, these results

support the idea that the majority of TM caused by these lesions results from TM occurring before IPTG induction.

Strand Breaks Cause TM in E. coli Deficient in AP Endonuclease Activity. We were also interested in strand breaks resulting from AP lyase–mediated β,δ elimination. As above, we compared the TM that resulted from bypass of a single lesion (strand break) between strains of different DNA repair backgrounds and determined the role of different repair enzymes in the repair of that lesion. We found that strand breaks do cause TM, with *nfo* mutants displaying increased TM compared to xth mutants (Table 2). These results demonstrate that the 3′-phosphatase activity of Nfo exerts a greater effect in prevention of strand break–mediated TM than that of Xth. Simultaneous removal of both AP endonucleases resulted in a further increase in TM for all time points examined (Table 2 and Figure 4A), and, similar to TM caused by AP sites, the majority of TM caused by strand breaks appears to be occurring during the 30 minute recovery, as there was not a substantial increase in TM following IPTG induction (Figures 3B and 4A). We observed that Nfi cannot prevent strand break-mediated TM (Table 2 and Table 3). Collectively, these results demonstrate DNA strand break bypass by RNAP, resulting in TM.

RNAP Bypass of Strand Breaks Results in Production of In-Frame Mutant Transcripts. The above findings revealed a significant in vivo role for AP endonucleases in processing 3′ blocking groups and preventing TM caused by strand breaks. Nevertheless, the observation of high levels of luciferase activity was unexpected, as this would require the production of high levels of full-length, in-frame luciferase mRNA. Previous in vitro studies demonstrated that strand breaks with 3′- and

5′-phosphate termini are not bypassed efficiently by prokaryotic RNAPs compared to other types of strand breaks (10-14). In addition, the transcripts resulting from in vitro break/gap bypass contain deletions equal to the size of the gap (10-14). Our cDNA sequencing revealed that RNAP bypass of strand breaks containing phosphate termini in vivo resulted in adenine incorporation, but no deletions, in the resulting transcripts (Figure 4B). This result was completely unexpected and indicates that there are factors that aid RNAP in the bypass of strand breaks and gaps in vivo. However, the level of bypass is likely to be low compared with certain types of base damage. Accordingly, the 8OG/ST construct yielded higher TM levels than the SSB/ST construct (Figure 4A and Table 2), most likely due to RNAP bypass of 8-oxoguanine occurring before conversion in vivo to a SSB via BER. This is supported by the results of 8OG/ST cDNA sequencing in *xth nfo nfi* cells, which revealed cytosine incorporation. Adenine and cytosine insertion events were previously observed using the 8OG/ST construct in glycosylase (MutM)–deficient strains (3).

DISCUSSION

This study provides an in vivo demonstration of RNAP bypass of AP sites and strand breaks. In addition, through the use of a panel of *E. coli* strains deficient in various DNA repair enzymes, we were able to elucidate the relative roles of various repair enzymes in nondividing cells.

We demonstrated that Nfo plays a greater repair role than Xth for the processing of both AP sites and 3′ blocking groups in cells in a nongrowth state. Xth has generally been believed to be the major AP endonuclease in *E. coli*, whereas Nfo plays a more minor role; but here we show that Nfo is more important for the prevention of TM caused by BER intermediates. However, Xth is still important, as simultaneous removal of both Xth and Nfo resulted in an increase in TM compared with Nfo alone.

Once it was known that both of the lesions examined here could be bypassed by RNAP, it was important to determine the nucleotide that RNAP incorporated opposite the lesions and to determine whether there were any deletions in the transcript as had been observed in vitro. RNAP appears to follow the same "A-rule" of the DNA polymerases, wherein the polymerase will preferentially incorporate adenine opposite noninformative sites on the template strand (22).

It was surprising that when RNAP bypassed the gapped, strand break structure used in this study, there were never any deletions observed in the transcript population. In vitro, for all combinations of strand break ends observed, the resulting transcripts contained a deletion in the mRNA (10-14). Our results demonstrate that there are factors that facilitate RNAP bypass of broken/gapped templates in vivo to prevent production of

mRNA containing frameshift mutations, and that strand breaks are an additional noninformative lesion that follows the "A-rule" for polymerase bypass. In addition, these data demonstrate the importance of performing TM studies in live cells, as there are clearly many factors that can affect the bypass of lesions. It will be interesting to examine strand break bypass in eukaryotic cells, as human RNAP II can also bypass gapped strand breaks with various ends in vitro, albeit at a low efficiency (23).

Although RNAP can bypass strand breaks without introducing a deletion into the transcript, it is likely that the rate of bypass is low compared with other types of base damage. The levels of luciferase, and therefore TM, observed are mediated by several factors, including the rates of repair and the level of bypass. Although the TM-LAS does not allow us to independently measure these two factors, the transcript sequencing results allow several inferences to be made. Transcript sequencing revealed that the processing of strand breaks containing a 3′-phosphate terminus is slow compared with AP site processing and should therefore lead to higher levels of TM caused by strand breaks than that observed from AP sites. However, TM is approximately equal for these two lesions (Figures 2A and 4A and Table 2), indicating that the rates of RNAP bypass of strand breaks must be low compared with those in the AP sites, effectively lowering the total amount of luciferase transcript that can be produced and lowering TM to the level observed for AP sites. Unfortunately, *E. coli* possess robust activities to remove truncated mRNAs from the transcript pool (24), preventing a direct measurement of the in vivo RNAP bypass and stalling at this time.

Transcript sequencing information allowed us to make several conclusions about repair of AP sites and strand breaks in vivo. Sequencing was carried out in *xth nfo nfi*

DNA repair mutant strains. The difference in repair of AP sites versus AP^{THF} indicates a role for AP lyases in the repair of AP sites, even if this activity is uncoupled from the glycosylase activity. Most surprising was the observation that repair of AP^{THF} can occur even in the absence of AP lyase activity, AP endonucleases, and Nfi with nearly complete repair observed soon after the 30 minute recovery period following transformation. We have also shown that NER is not involved in the repair of AP^{THF}, indicating that an additional mechanism of repair exists. It is interesting to note that, although it would have been especially informative to have conducted TM studies with AP^{THF} in a strain that is deficient in *xth nfo nfi* and *uvrA* concurrently, thus compromising all known AP site repair pathways simultaneously, we were unable to construct such a quadruple mutant strain, even when using a P1 stock that produced viable mutants in other backgrounds (Table 1). Together, these results support recent observations that repair of AP sites can occur through another, unknown mechanism in addition to those mediated by AP endonucleases, AP lyases, NER, or Nfi (25). Similarly, the strand breaks are processed in the absence of AP endonucleases, Nfi, and
NER. It is known that nucleoside diphosphate kinase (NDK) possesses 3′-phosphatase activity, but a role in BER has not been demonstrated (26). A comparison of the transcript sequencing results of AP^{THF} and strand breaks demonstrates that the repair activity for strand breaks in these strains occurs at a somewhat lower level compared with that of the AP sites.

In the present study, we demonstrate that RNAP bypass of both AP sites and strand breaks can cause TM in vivo. As depicted in Figure 5, both nonbulky base lesions, and the intermediates that result from BER, are bypassed by RNAP resulting in TM.

Therefore, perturbation of any one of several steps of BER can increase the level of TM in a cell. There are several important biological consequences for TM (5). TM could be a mechanism for adaptive mutagenesis wherein the generation of a large mutant mRNA pool and subsequent accumulation of mutant proteins could alter the cellular phenotype. If such mutant proteins conferred a growth advantage, the altered phenotype could then become permanent during the ensuing round of DNA replication past the lesion in a process termed retromutagenesis (27). In bacteria and other unicellular organisms, retromutagenesis could result in escape from growth restricted environments, and/or the acquisition of antibiotic resistance.

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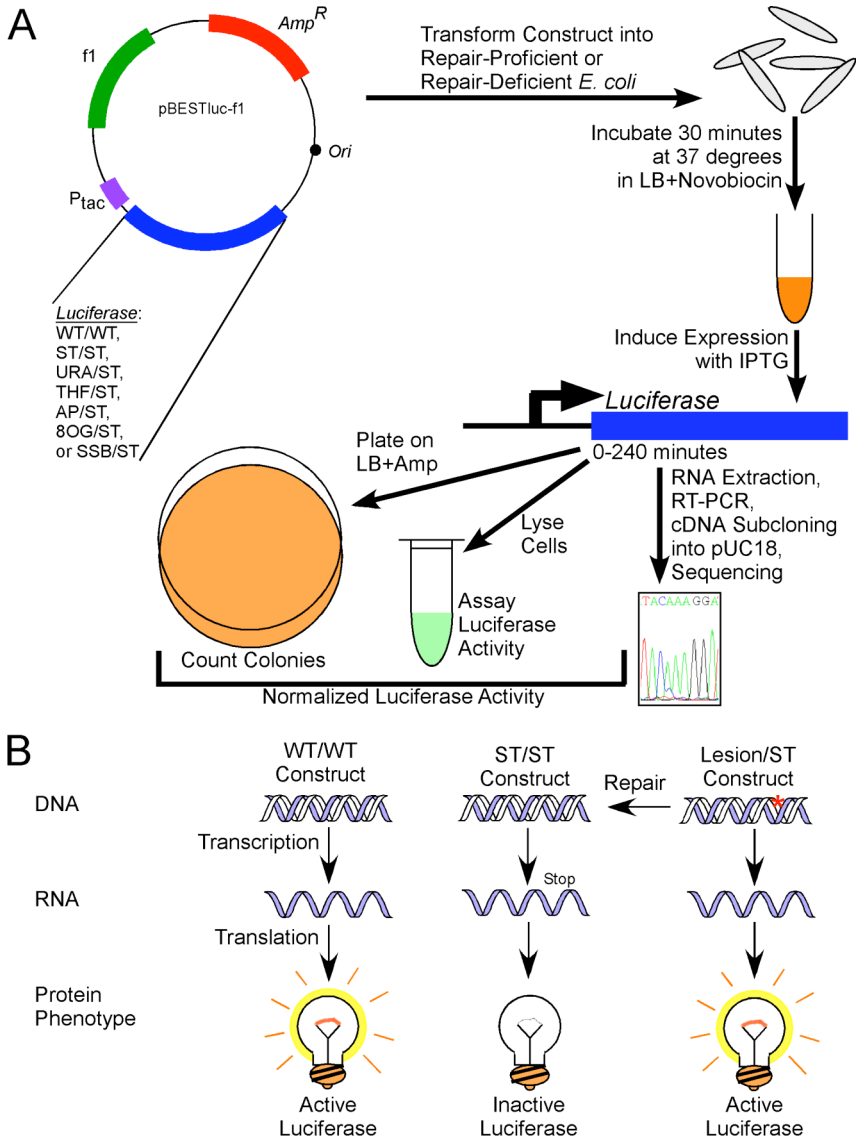
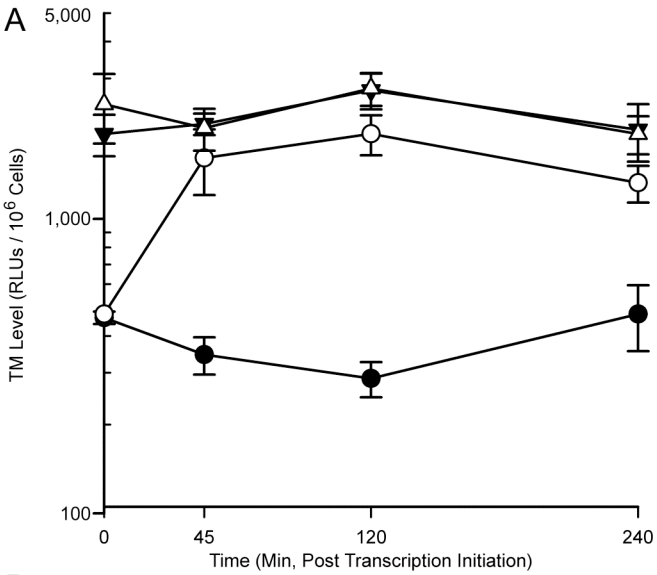


Figure 1. Transcriptional mutagenesis luciferase assay system (TM-LAS). (A) The pBESTluc-f1 luciferase reporter construct contains (i) the firefly luciferase gene (blue) driven by the E. coli tac promoter (purple); (ii) the ampicillin-resistance gene (red); (iii) the f1 origin of replication (green) for the production of single-stranded DNA corresponding to the coding strand of the luciferase gene; and (iv) an origin of double-strand DNA replication (Ori) to allow for its propagation. At codon 445 of the luciferase gene, sequence is either WT/WT (Norm/Norm, wild-type sequence of codon 445, AAA on the coding strand), ST/ST (codon 445 changed to Stop codon, TAA on coding strand), or Lesion/Stop (first base position of codon 445 in noncoding strand contains a lesion, XTT; coding strand is TAA Stop codon). Construct preparation has been described previously (3, 28). Constructs were electroporated into repair-proficient and -deficient E. coli cells subsequently incubated in LB medium containing novobiocin (to suppress growth) for 30 min. Luciferase gene expression was induced by the addition of IPTG (1 mM final) in the medium. At different times following luciferase induction (0–240 min) aliquots of cells were lysed, luciferase activity determined, and cell aliquots plated (LB-Amp) to normalize luciferase activity. Additionally, at different times following luciferase induction, RNA was isolated and subjected to RT-PCR and subcloning into pUC18 for cDNA sequencing. (B) Predicted results of luciferase assay for luciferase expressed from WT/WT (Norm/Norm), ST/ST, Lesion/ST constructs. WT/WT (Norm/Norm) will produce full-length, wild-type luciferase enzyme, resulting in robust levels of luciferase activity. ST/ST will produce a truncated, inactive luciferase enzyme (3). Lesion/ST will produce full-length, active luciferase

only when RNAP bypasses the lesion and inserts a nucleotide other than uracil opposite the lesion. In the event of repair, the thymine in the nontemplate strand will specify adenine incorporation on the template strand during DNA repair synthesis (Figure 2). Adenine will code for uracil in the mRNA transcript, resulting in a Stop codon and a truncated, inactive luciferase (29). Therefore, a luciferase activity (RLUs) level produced from the Lesion/ST construct that is greater than the ST/ST control construct will indicate RNAP bypass, with the occurrence of TM verified by transcript cDNA sequence analysis.



B

DNA 5′...TAC TAA GGA...3′ Repair 5′...TAC TAA GGA...3′
3′...ATG _TT CCT...5′ 3′...ATG ATT CCT...5′
Transcriptional Mutagenesis
RNA 5′...UAC AAA GGA...3′ 5′...UAC UAA GGA...3′
(Lys) (Stop)
(Active Luciferase) (Inactive Luciferase)
cDNA 5′...TAC AAA GGA...3′ 5′...TAC TAA GGA...3′
3′...AGT TTT CCT...5′ 3′...ATG ATT CCT...5′

Damaged Introduced (Time Post Induction)	Ribonucleotide Incorporated and No. of Insertion Events Detected	
	Adenine	Uracil
AP/ST (5 min*)	123 (100%)	
AP/ST (1 min)		89 (100%)
AP/ST (2 min)		63 (100%)
AP^{THF}/ST (1 min)	11 (11.5%)	85 (88.5%)
AP^{THF}/ST (2 min)	18 (16.5%)	91 (83.5%)
AP^{THF}/WT (2 min)	45 (100%)	
URA/ST (1 min)	65 (69.1%)	29 (30.9%)
URA/ST (2 min)	102 (100%)	
URA/ST (15 min)		59 (100%)

Figure 2. AP site-mediated transcriptional mutagenesis in vivo. (A) AB1157 *xth nfo* cells were transformed with URA/ST (open circles), AP^{THF}/ST (closed triangles), AP/ST (open triangles), or ST/ST (closed circles) construct, allowed a 30-min recovery from electroporation, and TM level (expressed as relative light units or RLUs) was assayed by TM-LAS (Figure 1) at 0, 45, 120, and 240 min following IPTG initiation of transcription. Each point represents the mean of at least three replicates ± SEM. (B) AB1157 xth nfo nfi cells were transformed with designated damage-containing constructs, followed by a 30-min recovery from electroporation; then, at indicated times following luciferase induction, RNA was extracted to determine AP site-driven TM events. *In the AP/ST 5-min experiment, cells were immediately resuspended in medium containing IPTG following electroporation, and RNA was isolated 5 min later. RT-PCR and subsequent cDNA sequencing allowed determination of the ribonucleotide incorporated during RNAP bypass. The number of insertion events detected for each ribonucleotide is indicated for either adenine or uracil, with the percentage of the total in parentheses. Each number represents combined sequencing results from two independent transformation events with two independent preparations of construct. Uracil at the first position of codon 445 results from transcription of repaired molecules and yields a Stop codon (producing inactive luciferase), except in the case of AP^{THF}/WT, in

which repaired constructs would direct adenine insertion at the first position of codon 445. Adenine insertion results from RNAP bypass over the AP site and yields a Lys codon (producing active luciferase).

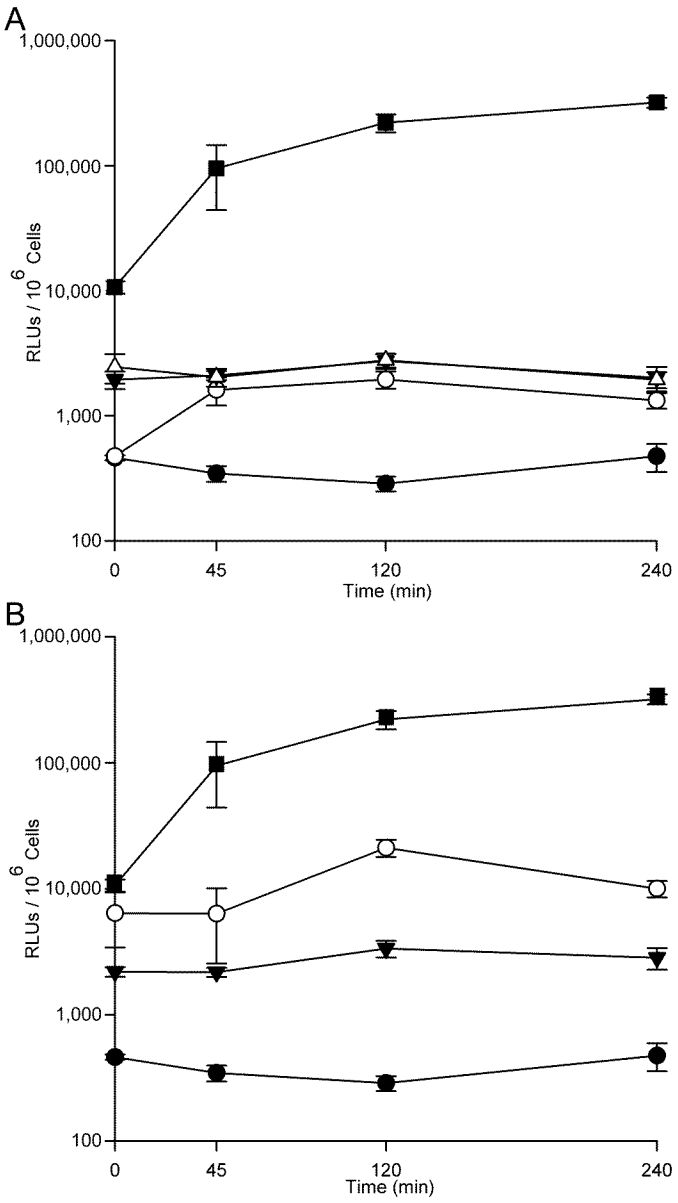
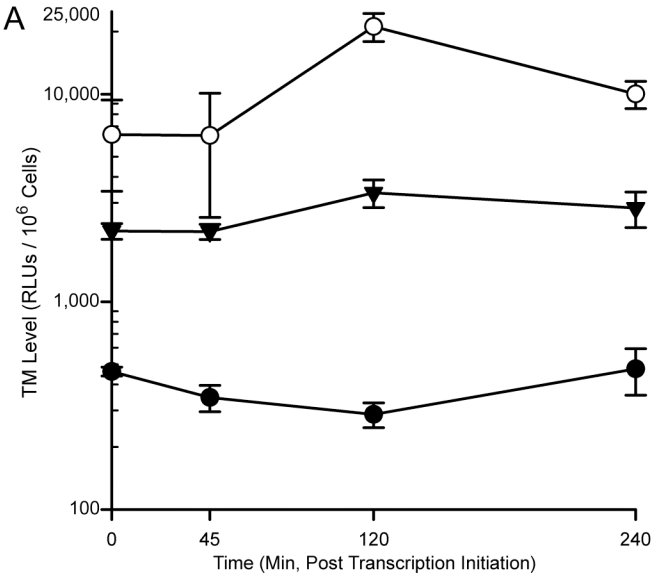
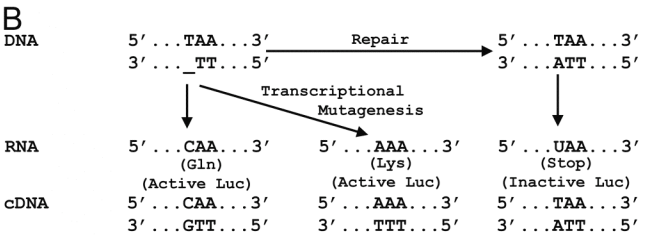


Figure 3. Observed leakiness of the luciferase promoter used in these studies. (A) AB1157 *xth nfo* cells were transformed with URA/ST (open circles), AP^{THF}/ST (closed triangles), AP/ST (open triangles), ST/ST (closed circles) or Norm/Norm (closed squares) construct, allowed a 30 minute recovery from electroporation, and TM level (expressed as relative light units or RLUs) was assayed by TM-LAS (Figure 1) at 0, 45, 120 and 240 min following IPTG initiation of transcription. Each point represents the mean of at least three replicates ± SEM. (B) AB1157 *xth nfo* cells were transformed with 8OG/ST (open circles), SSB/ST (closed triangles), ST/ST (closed circles), or Norm/Norm (closed squares) construct, allowed a 30 min recovery from electroporation, and TM level (expressed in relative light units or RLUs) for these transformed cells was measured as before (Figure 2A). The high levels of luciferase activity observed at t = 0 in panels A and B indicate some leakiness of transcription in the absence of IPTG induction.





Damage Introduced (Time Post Induction)	Ribonucleotide Incorporated and No. of Insertion Events Detected		
	Cytosine	Adenine	Uracil
8OG/ST (5 min)	133 (100%)		
8OG/ST (15 min)	38 (80.9%)		9 (19.1%)
SSB/ST (5 min)		41 (44.6%)	51 (55.4%)
SSB/ST (15 min)		47 (39.5%)	72 (60.5%)

Figure 4. Strand break–mediated transcriptional mutagenesis in vivo. (A) AB1157 *xth nfo* cells were transformed with 8OG/ST (open circles), SSB/ST (closed triangles) or ST/ST (closed circles) construct, allowed a 30 minute recovery from electroporation, and TM level (expressed in relative light units or RLUs) for these transformed cells was measured as before (Figure 2). (B) AB1157 *xth nfo nfi* cells were transformed with indicated damage-containing constructs, and RNA extracted to determine strand break-driven TM as before (Figure 2).

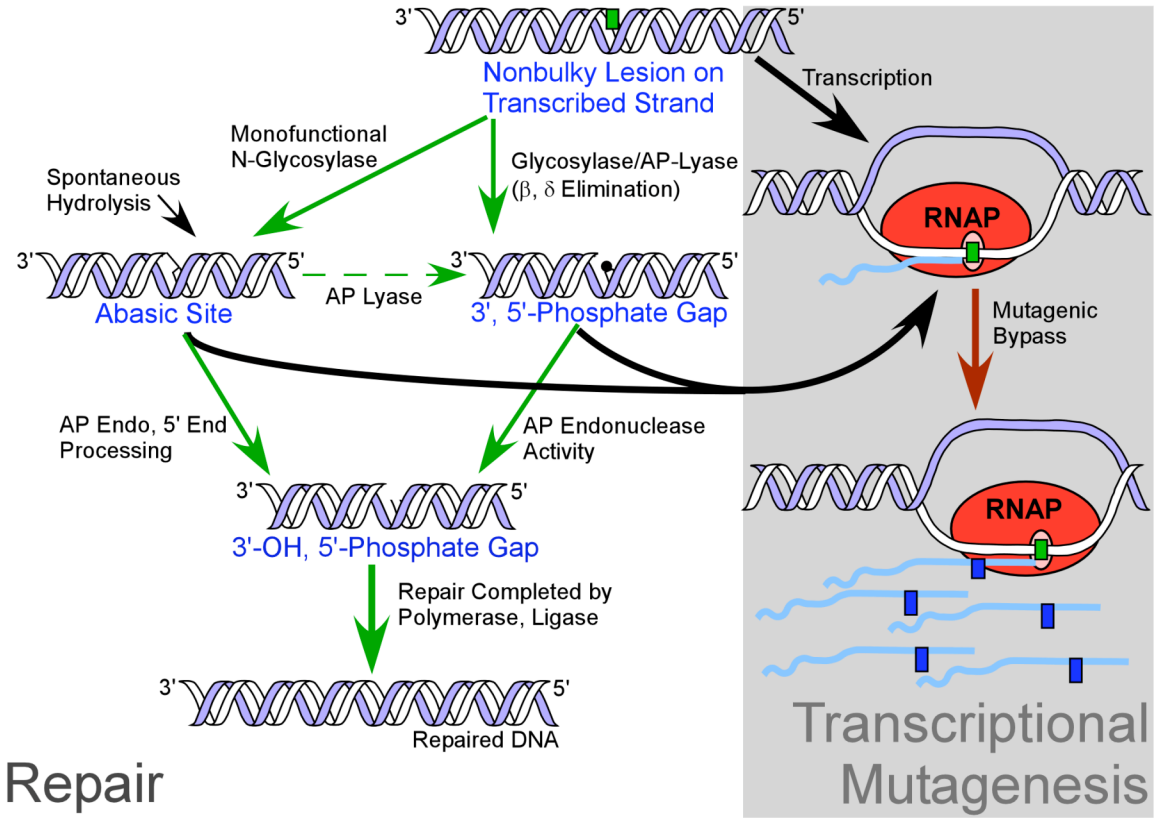


Figure 5. RNAP bypass of AP sites and strand breaks reveal the relationship between the transcriptional machinery and DNA repair processes. Nonbulky lesions (small green box) in DNA cause transcriptional mutagenesis (TM) and are primarily repaired by enzymes in the base excision repair (BER) pathway. DNA N-glycosylases remove base lesions from the DNA, generating an abasic site (monofunctional glycosylase) or a strand break (bifunctional glycosylase/AP-lyase). Such BER intermediates also cause TM, where RNAP bypass results in transcripts containing adenine inserted opposite the site of damage. Repair of these BER intermediates can be mediated by AP endonucleases, and the absence of AP endonucleases will significantly increase TM caused by BER intermediates.

Table 1: Strains and Primers Used in This Study

Strains	Description	Source
AB1157	*thr-1 araC14 leuB6 (Am) Δ(gpt-pro) lacY1 tsx-33 qsr'-0 glnV44 (AS) galK2 (Oc) Rac-0 hisG4 (Oc) rfbD1 mgl-51 rpoS396 (Am) rpsL31 kdgK51 xylA5 myl-1 argE3 (Oc) thi-1*	B. Weiss collection (30)
BW527	AB1157 *nfo-1::kan*	B. Weiss collection (31)
BW528	AB1157 *nfo-1::kan Δ(xth-pncA)*	B. Weiss collection (31)
BW804	*[araD139] ptsF25 Δ(argF-lac)205 flbB5301 relA1 rpsL150 deoC1 rbsR λ Φ(nfo'-lac) cI857 nin5 Ap Km*	B. Weiss collection (32)
BW1161	AB1157 *nfi-1::cat*	B. Weiss collection (16)
BW1163	AB1157 *nfi-1::cat Δ(xth-pncA)90 nfo-1::kan*	B. Weiss collection (16)
BW1743	AB1157 *uvrA::Tn10*	B. Weiss collection
BW9109	AB1157 *Δ(xth-pncA)90*	B. Weiss collection (33)
DH12S	*φ80dlacZM15 mcrA Δ(mrr-hsdRMS-mcrBC) araD139 Δ(ara, leu)7697 ΔlacX74 galU galK rpsL deoR nupG recA1 [F'proAB+ lacIqZΔM15]*	P. Doetsch collection
Z104	AB1157 *uvrA::Tn10 nfo-1::kan*	P1(BW1743) x BW527
Z107	AB1157 *nfi-1::cat nfo-1::kan*	P1(BW1161) x BW527
Primers	Sequence	
Const-Normal	5′phos-CGATTCCAATTAAGCGGGGGCCACCTGATATCCTTTGTATTTAAT-3′	
Const-Stop	5′phos-CGATTCCAATTAAGCGGGGGCCACCTGATATCCTTAGTATTTAAT-3′	
Const-Ura (U)	5′phos-CGATTCCAATTAAGCGGGGGCCACCTGATATCCTTUGTATTTAAT-3′	
Const-THF (F)	5′phos-CGATTCCAATTAAGCGGGGGCCACCTGATATCCTTFGTATTTAAT-3′	
Const-8OG (8)	5′phos-CGATTCCAATTAAGCGGGGGCCACCTGATATCCTT8GTATTTAAT-3′	
Nfi1	5′-ATGGATCTCGCGTCATTAC-3′	
Nfi2	5′-CAGTTTACCTGAATTAGGG-3′	
LBRT1	5′-TTGACTGGCCACGTAATCC-3′	
LBRT2	5′-GACCAACGCCTTGATTGAC-3′	
Clo18U	5′-GCTGCAAGGGGATTAAGTT-3′	
Clo18L	5′-CGGGTCGTATGTTGTGTGG-3′	

Table 2: TM Caused by Abasic Sites and Strand Breaks in Repair-Proficient and Deficient *E. coli*

Construct, Strain*	RLU[†]/10^6 cells ± SEM[‡]	Mutant/Wild-Type[§]	p-Value[¶]
URA/ST	TS[‖] 3′ ATG **U**TT CCT 5′		
	NTS** 5′ TAC TAA GGA 3′		
AB1157 (WT)	193 ± 32[††]		
AB1157 *xth*	207 ± 47	1.07	0.8057
AB1157 *nfo*	395 ± 40	2.04	0.00081
AB1157 *xth nfo*	1946 ± 302	10.07	0.0000031
AB1157 *xth nfo nfi*	641 ± 58	3.32	0.0000014
AP^{THF}/ST	TS[‖] 3′ ATG **F**TT CCT 5′		
	NTS** 5′ TAC TAA GGA 3′		
AB1157 (WT)	585 ± 63		
AB1157 *xth*	363 ± 113	0.62	0.0890
AB1157 *nfo*	1999 ± 285	3.42	0.00014
AB1157 *xth nfo*	2733 ± 378	4.67	0.000012
AB1157 *xth nfo nfi*	1409 ± 173	2.41	0.00014
AP/ST	TS[‖] 3′ ATG _TT CCT 5′		
	NTS[**] 5′ TAC TAA GGA 3′		
AB1157 (WT)	371 ± 243		
AB1157 *xth*	346 ± 100	0.93	0.9337
AB1157 *nfo*	1104 ± 222	2.98	0.0473
AB1157 *xth nfo*	2774 ± 355	7.48	0.00032
AB1157 *xth nfo nfi*	874 ± 217	2.36	0.1662
8OG/ST	TS[‖] 3′ ATG **8**TT CCT 5′		
	NTS** 5′ TAC TAA GGA 3′		
AB1157 (WT)	410 ± 135[††]	1.00	
AB1157 *xth*	194 ± 94	0.47	0.2742
AB1157 *nfo*	3015 ± 575	7.35	0.0013
AB1157 *xth nfo*	21160 ± 3261	51.56	0.000083
AB1157 *xth nfo nfi*	7773 ± 1292	18.94	0.00021
SSB/ST	TS[‖] 3′ ATG //TT CCT 5′		
	NTS** 5′ TAC TAA GGA 3′		
AB1157 (WT)	87 ± 22		
AB1157 *xth*	78 ± 6	0.90	0.6946
AB1157 *nfo*	1523 ± 697	17.50	0.0847
AB1157 *xth nfo*	3351 ± 510	38.51	0.000057
AB1157 *xth nfo nfi*	770 ± 344	8.85	0.000048

*Full strain descriptions in Table 1.

[†]Relative light units measured 120 min post IPTG induction.

[‡]Each value is the average of at least three replicate samples ± standard error of the mean.

[§]Ratio of mutant luciferase activity over repair-proficient luciferase activity for the same construct.

[¶]p-values for Student's t-test comparison between mutant and repair-proficient luciferase activity for the same construct. Distributions were considered to be significantly different when $p < 0.01$.

[‖]Transcribed strand. U=uracil, F=tetrahydrofuran, _=abasic site, 8=8-oxoguanine, //=strand break

**Nontranscribed strand

††This data included in a previously submitted manuscript

Table 3: TM Caused by Abasic Sites and Strand Breaks in *E. coli*, Including Control Constructs

Construct/Strain*	RLU[†]/106 cells ± SEM[‡]	Mutant/Wild-Type[§]	p-Value[¶]
Norm/Norm (WT)	TS[‖] 3′ ATG TTT CCT 5′ NTS** 5′ TAC AAA GGA 3′		
AB1157 (WT)	228898 ± 155629[††]	1.00	
AB1157 *xth*	313168 ± 8380	0.14	0.3899
AB1157 *nfo*	178330 ± 32141	0.78	0.9458
AB1157 *xth nfo*	220329 ± 36248	0.96	0.9791
AB1157 *xth nfo nfi*	380113 ± 29069	1.66	0.5100
AB1157 *nfi*	79808 ± 12359	0.35	0.6492
AB1157 *uvrA*	218286 ± 45841[††]	0.95	0.9484
AB1157 *nfo uvrA*	228469 ± 66456	1.00	0.9985
AB1157 *nfo nfi*	121967 ± 19133	0.53	0.7438
ST/ST	TS[‖] 3′ ATG ATT CCT5′ NTS** 5′ TAC TAA GGA 3′		
AB1157 (WT)	120 ± 39[††]	1.00	
AB1157 *xth*	90 ± 13	0.75	0.6169
AB1157 *nfo*	287 ± 39	2.40	0.3341
AB1157 *xth nfo*	182 ± 47	1.52	0.0127
AB1157 *xth nfo nfi*	360 ± 144	3.00	0.1393
AB1157 *nfi*	280 ± 48	2.34	0.0437
AB1157 *uvrA*	213 ± 73[††]	1.78	0.2861
AB1157 *nfo uvrA*	308 ± 93	2.58	0.0771
AB1157 *nfo nfi*	114 ± 41	0.96	0.9349
URA/ST	TS[‖] 3′ ATG **U**TT CCT 5′ NTS** 5′ TAC TAA GGA 3′		
AB1157 (WT)	193 ± 32[††]	1.00	
AB1157 *nfi*	179 ± 14	0.93	0.2912
AB1157 *nfo uvrA*	498 ± 50[‡‡]	2.58	0.00018
AB1157 *nfo nfi*	1208 ± 65[§§]	6.25	0.000000000090
AP^{THF}/ST	TS[‖] 3′ ATG **F**TT CCT5′ NTS** 5′ TAC TAA GGA 3′		
AB1157 (WT)	585 ± 63	1.00	
AB1157 *uvrA*	94 ± 18	0.16	0.000050
AB1157 *nfo uvrA*	309 ± 61[§§]	0.53	0.0123
AB1157 *nfo nfi*	360 ± 86[§§]	0.62	0.0709
AP/ST	TS[‖] 3′ ATG _TT CCT 5′ NTS** 5′ TAC TAA GGA 3′		
AB1157 (WT)	371 ± 243	1.00	
AB1157 *uvrA*	683 ± 87	1.84	0.2543
AB1157 *nfo uvrA*	1083 ± 136[‡‡]	2.92	0.0283
AB1157 *nfo nfi*	212 ± 38[§§]	0.57	0.5322
8OG/ST	TS[‖] 3′ ATG **8**TT CCT 5′ NTS** 5′ TAC TAA GGA 3′		

AB1157 (WT)	410 ± 135[††]	1.00	
AB1157 *nfo uvrA*	3078 ± 395[‡‡]	7.50	0.00011
AB1157 *nfo nfi*	2015 ± 132[‡‡]	4.91	0.0000018
SSB/ST	TS[‖] 3′ ATG //TT CCT 5′ NTS** 5′ TAC TAA GGA 3′		
AB1157 (WT)	87 ± 22	1.00	
AB1157 *uvrA*	162 ± 39	1.86	0.1221
AB1157 *nfo uvrA*	352 ± 39[‡‡]	4.04	0.00014
AB1157 *nfo nfi*	157 ± 17[‡‡]	1.80	0.0299

*Full strain descriptions in Supplementary Table 1.

[†]Relative light units measured 120 min post IPTG induction.

[‡]Each value is the average of at least three replicate samples ± standard error of the mean.

[§]Ratio of mutant luciferase activity over repair-proficient luciferase activity for the same construct.

[¶]p-values for Student's t-test comparison between mutant and repair-proficient luciferase activity for the same construct. Distributions were considered to be significantly different when $p < 0.01$.

[‖]Transcribed strand. U=uracil, F=tetrahydrofuran, _=abasic site, 8=8-oxoguanine, //=strand break

**Nontranscribed strand

[††]This data included in previously submitted manuscript

[‡‡]p-value > 0.01 for t-test comparison between listed strain and *nfo* strain

[§§]p-value < 0.01 for t-test comparison between listed strain and *nfo* strain

CHAPTER 4

DISCUSSION AND FUTURE DIRECTIONS

DNA must faithfully carry all of the genetic information for an organism, and yet it is constantly subject to damage and modification. This can occur through several mechanisms, including hydrolysis, oxidation and methylation (1). During replication in dividing cells, unrepaired damage can lead to mutations by directing the incorporation of incorrect bases into the newly synthesized DNA strand. While cells in a laboratory setting are nurtured and grow and divide often, most cells in nature are neither dividing constantly, nor undergoing continuous rounds of replication (2). Therefore, cellular vigor is largely dependent on faithful transcription and translation, not replication. RNA polymerase (RNAP) encounters with DNA damage can result in one of three outcomes: (i) RNAP blockage and transcriptional arrest, (ii) initiation of transcription-coupled repair (TCR) and removal of the lesion, or (iii) RNAP bypass of the lesion in some cases resulting in transcriptional mutagenesis (TM).

The work in this dissertation delineates the ways in which DNA repair pathways can mediate TM *in vivo*. We have demonstrated that: (1) TCR can initiate repair of non-bulky base lesions *in vivo* allowing for dynamic flexibility for the repair of lesions known to cause TM. Specifically, the frequently occurring, biologically important lesions 8-oxoguanine and uracil were shown to be substrates for TCR. (2) During TCR of non-bulky base damage in the DNA, both nucleotide excision repair (NER) and base excision repair (BER) are utilized during the completion of repair. This is especially true for 8-oxoguanine, and we hypothesize that this is because the opposite base specificity offered by the BER glycosylases that recognize 8-oxoguanine (MutM and MutY) would provide a greater opportunity for error-free repair of the lesion. (3) Repair intermediates such as strand breaks and abasic sites are bypassed by RNAP *in vivo*. (4) The RNAP bypass of

strand breaks and abasic sites can be mutagenic, causing (TM). We consider these findings to be a significant contribution to the understanding of the process of TM and DNA repair *in vivo* and we hypothesize that many more lesions will be found to be subject to both TM and TCR, and that the balance between TM and repair is important for the health of individual cells and whole organisms.

***Dynamic flexibility of DNA repair pathways in the removal of non-bulky damage from DNA in* Escherichia coli.**

The data presented in Chapter 2 demonstrate a dynamic flexibility in the removal of non-bulky DNA lesions, uracil and 8-oxoguanine, from DNA. TCR and NER are important for the removal of both of these lesions from DNA.

The data in Chapter 2 shows that TCR acts directly on 8-oxoguanine and uracil, and not on BER intermediates such as abasic sites or strand breaks. This conclusion can be drawn by a comparison of mutagenesis caused by 8-oxoguanine and uracil in a strain deficient in a single repair protein in TCR (Mfd), strains deficient in the specific BER glycosylase for that lesion, MutM or Ung, respectively, and strains that are missing both the glycosylase and TCR, *mutM mfd* and *ung mfd*, respectively. By removing the major DNA glycosylase, the initiating lesion remains in the DNA for a prolonged period of time and repair intermediates resulting from DNA glycosylase-mediated removal will be produced more slowly (3). If Mfd recognizes repair intermediates instead of 8-oxoguanine or uracil directly, no increase in mutagenesis would be observed in *ung mfd* or *mutM mfd* double mutants compared to the *ung* or *mutM* mutant alone because there would be fewer repair intermediates to direct recruitment of Mfd and mutagenesis would

be unchanged or reduced. But when loss of the glycosylase was combined with elimination of Mfd, the increase in mutagenesis was synergistic, revealing a role of Mfd-mediated TCR in the removal of uracil and 8-oxoguanine (Chapter 2, Figure 2A, 2D and Table 2). Therefore, despite the fact that both uracil and 8-oxoguanine are primarily repaired by BER, the data included in Chapter 2 indicate that TCR is able to aid in the removal of these frequently occurring lesions, suggesting that dynamic flexibility of repair may be a general feature of several types of base damage.

Previous data indicated that there is a great degree of overlap of repair by BER, NER, recombination repair and translesion synthesis in replicating yeast (4, 5). We therefore wanted to examine the role of NER in the repair of both uracil and 8-oxoguanine in *E. coli*. NER could be involved in the repair of non-bulky lesions through direct recognition, or via initiation by TCR (Mfd), as discussed above. We observed that in strains lacking UvrA, the initiating protein of the NER pathway, there was a significant increase in mutagenesis caused by uracil compared to that in repair-proficient cells upon induction of transcription (Chapter 2, Figure 2B, 2C, and Table 2), confirming a role of NER in the removal of uracil from DNA. Additionally, use of a *uvrA mfd* double mutant revealed a direct role for NER of uracil, independent of Mfd-mediated TCR (Chapter 2, Figure 2C and Table 2). These results were surprising since NER is suspected to recognize helix distortion by bulky DNA damage (6), and uracil in DNA would not cause significant helix distortion. However, because NER does not require the lesion recognition specificity characteristic of the N-glycosylases of the BER pathway, this could make NER especially flexible for the repair of many types of lesions, even those that are considered to be helix non-distorting.

For 8-oxoguanine we observed that removal of NER resulted in an increase in mutagenesis, indicating that NER is likely involved in the repair of 8OG *in vivo* (Chapter 2, Figure 2D, 2E and Table 2). Again, this result was surprising since 8OG is not known to cause any helix distortion. These results would indicate that the dynamic flexibility of DNA repair pathways is important for multiple frequently occurring non-bulky base damages. The data in Chapter 3 (Table 3), as well as work from others (7), confirms that these data do not represent NER of repair intermediates, such as abasic sites and single strand breaks, but rather NER of uracil and 8-oxoguanine directly.

Interestingly, the data from examination of TM caused by 8-oxoguanine revealed the possibility that TCR uses both NER and BER. If repair of 8-oxoguanine initiated by Mfd occurred only through the NER pathway, *mfd* should be epistatic to *uvrA*, and there should be no elevation of mutagenesis in the *mfd uvrA* double mutant compared to the *uvrA* mutant. Unexpectedly though, there was a significant increase in mutagenesis in the double mutant compared to the *uvrA* mutant, demonstrating that not all repair initiated by Mfd is mediated by UvrA (NER pathway), thus substantiating a role for BER components in TCR. This was a completely unexpected result as TCR proceeding through a BER mechanism has not been demonstrated previously in any organism.

We proposed that BER may be the preferred method of TCR of these lesions due to its opposite base specificity. 8-oxoguanine can pair with adenine as well as with cytosine, so cells have evolved two N-glycosylase enzymes for the processing of this lesion. MutM, discussed above, removes 8-oxoguanine from the DNA best when the 8-oxoguanine is paired with a cytosine residue, but has little activity when 8-oxoguanine is opposite an adenine residue (8). If MutM were to remove the 8-oxoguanine when it is

paired with adenine, the DNA polymerase would put in a thymine residue opposite the adenine during repair synthesis, resulting in a permanent guanine to thymine transversion mutation. Therefore, when 8-oxoguanine is paired with adenine, a separate N-glycosylase, MutY, removes the adenine from the DNA. This gives the repair DNA polymerase an opportunity to put in the non-mutagenic base, cytosine, opposite the 8-oxoguanine before the lesion is removed and the DNA repaired (8). NER lacks this opposite base specificity, such that if NER were used to remove 8-oxoguanine from the DNA instead of BER, it could result in increased mutagenesis. For this reason the cell likely prefers to use BER during TCR than NER. Interestingly, there is a synergistic increase in mutagenesis for both uracil and 8-oxoguanine when TCR and BER are eliminated simultaneously (Chapter 2 Figure 2A, Table 2, and (9)), reflecting a degree of competition between these two pathways. Based on these observations, we proposed that TCR can utilize either NER or BER components for repair of non-bulky base damage, with BER as the predominant repair pathway (Chapter 2, Figure 3).

The dynamic flexibility of DNA repair of non-bulky lesions provides cells with a greater capacity for repair, especially if one repair pathway is compromised, or under conditions where the capacity of a single pathway has been exceeded by the level of DNA damage. Such a situation could occur when a cell is exposed to a DNA damaging agent, resulting in a variety of different DNA lesions introduced into the genome at different levels, a subset of which may only be primarily repaired by a single pathway (e.g. BER). In the experiments presented in Chapter 2, through the use of *E. coli* strains deficient in DNA repair proteins, we were able to mimic a situation where one pathway is overwhelmed by damage and unavailable for use for repair of other lesions. We revealed

a role of TCR and NER in the repair of several frequently occurring, non-bulky lesions, a surprising result since these pathways were suspected to only be used for bulky, helix-distorting lesions. In particular, TCR seems to be able to proceed via either BER or NER, making the repair of damage in actively transcribed genes particularly flexible. As the majority of cells in nature are not actively dividing or replicating their DNA, flexibility of repair of active regions of the genome becomes particularly important. Though *in vitro* studies of RNAP demonstrate no stalling during bypass of many non-bulky lesions (10-12), it is possible, *in vivo*, the RNAP stalls infrequently, recruiting Mfd to the damage, thereby preventing further bypass by RNAP until repair can be completed. Therefore, the increase in TM observed in the absence of Mfd is relatively modest due to the low frequency of RNAP stalling, not because non-bulky lesions are poor substrates for TCR (Chapter 2, Figure 3).

Our results reveal a novel model of transcriptional encounters with non-bulky DNA damage (Chapter 2, Figure 3). Unrepaired lesions can be bypassed by RNAP, leading to transcriptional mutagenesis by insertion of an incorrect ribonucleotide opposite the lesion into the mRNA (9). Importantly, when RNAP stalls at the damage, Mfd can be recruited, initiating TCR. Mfd removes RNAP from the lesion and then acts as a placeholder, blocking incoming RNAP from bypassing the damage but leaving the lesion accessible to repair by BER. If BER capacity is exceeded, Mfd can recruit NER to complete repair of the lesion, compensating for the reduced capacity of BER. The discovery that NER components can be used for some level of U and 8OG repair is unexpected and reveals the dynamic flexibility of DNA repair pathway interrelationships *in vivo*.

Base excision repair intermediates such as abasic sites and single strand breaks can be bypassed by RNAP in* Escherichia coli*, resulting in transcriptional mutagenesis.

While 8-oxoguanine and uracil can be recognized and repaired by TCR, the predominant pathway for repair is BER. During the course of BER several repair intermediates, which are mutagenic themselves, are produced. We wanted to know if these repair intermediates would also cause TM *in vivo*. The data presented in Chapter 3 provides an *in vivo* demonstration of RNAP bypass of AP sites and strand breaks. Additionally, the use of a panel of *E. coli* strains deficient in various DNA repair enzymes allowed us to elucidate the relative roles of various repair enzymes in nondividing cells.

We demonstrated that one AP endonuclease in *E. coli*, Nfo, plays a greater repair role than another AP endonuclease, Xth, for the processing of both AP sites and 3′ blocking groups in cells in a non-growth state. (Chapter 3, Table 2). Although Xth has generally been believed to be the major AP endonuclease in *E. coli*, whereas Nfo plays a more minor role, the data presented in Chapter 3 demonstrate that Nfo is more important for the prevention of TM caused by BER intermediates. However, Xth is still necessary, as simultaneous removal of both Xth and Nfo resulted in an increase in TM compared with Nfo alone.

We also determined that RNAP appears to follow the "A-rule" of the DNA polymerases, wherein the polymerase will preferentially incorporate adenine opposite non-informative sites on the template strand (13) (Chapter 3, Figures 2B and 4B). Given the dramatic bias of AP site formation, with depurination occurring 100 times more

frequently than depyrimidination (14, 15), adenine incorporation opposite the more than 10,000 daily spontaneous depurinations would be exceedingly mutagenic (16, 17). It is more complicated to predict the mutagenicity of adenine incorporation opposite the single strand break used in these studies, as there is a wide range of base damages processed by the *E. coli* DNA glycosylase/AP lyase enzymes Nei and MutM (18). Nonetheless, we do know that substrates for MutM include ring-opened and oxidized purines, so very often, adenine incorporation would be mutagenic.

It is surprising that when RNAP bypassed the gapped strand break structure used in this study, there were no deletions observed in the transcript population. *In vitro*, for all combinations of strand break ends observed the resulting transcripts contained a deletion in the mRNA (19-23). The results in Chapter 3 demonstrate that there are factors that facilitate RNAP bypass of broken/gapped templates *in vivo* to prevent production of mRNA containing frameshift mutations, and that strand breaks are an additional non-informative lesion that follow the "A-rule" for polymerase bypass. While adenine may be a mutagenic insertion, depending on the original source of the strand break, frame shift mutations would always be mutagenic, so it is beneficial to the bacteria to prevent frameshifts in the mRNA at all costs. These data also demonstrate the importance of performing TM studies in live cells, as there are clearly many factors that can affect the bypass of lesions. These studies should be expanded to include *in vivo* studies using eukaryotic cells, because it has been demonstrated that human RNAP II can bypass gapped strand breaks with various ends *in vitro*, albeit at a low efficiency (24).

While the assay system used in the studies in Chapter 3 does not allow us to directly measure the bypass efficiency of the RNAP for different lesions, we were able to

make some inferences about the ability of RNAP to bypass BER repair intermediates by examining the transcript sequencing. This is because the levels of luciferase, and therefore TM, observed are mediated by several factors, including the rates of repair and the level of bypass. Transcript sequencing reveals that the processing of strand breaks containing a 3′-phosphate terminus is slow compared with AP site processing and should therefore lead to higher levels of TM caused by strand breaks than that observed from AP sites (Chapter 3, Figure 2B, 4B). Interestingly, TM is approximately equal for these two lesions (Chapter 3, Figure 2A, 4A and Table 2), indicating that the rates of RNAP bypass of strand breaks must be low compared with those of AP sites, effectively lowering the total amount of luciferase transcript that can be produced and lowering TM to the level observed for AP sites.

Additionally, the data presented in Chapter 3 allow us to make several conclusions about repair of AP sites and strand breaks *in vivo*. Transcript sequencing was carried out in *xth nfo nfi* DNA repair mutant strains. The difference in repair of AP sites versus AP^{THF}, an AP site analog that cannot be processed by AP lyases (25), indicated an important role for AP lyases in the repair of AP sites, even if this activity is uncoupled from the glycosylase activity. Most surprising was the observation that AP^{THF} can be repaired even in the absence of AP lyase activity, AP endonucleases, and Nfi. Since the data presented in Chapter 3 also indicate that NER is not involved in the repair of AP^{THF} (Chapter 3, Table 3), this implies the existence of an additional mechanism of repair that has yet to be discovered. Our results support observations that repair of AP sites can occur through another, unknown mechanism in addition to those mediated by AP endonucleases, AP lyases, NER, or Nfi (26). Similarly, the strand breaks are processed in

the absence of AP endonucleases, Nfi, and NER. It is known that nucleoside diphosphate kinase (NDK) possesses 3′-phosphatase activity, but a role in BER has not been demonstrated (27). Because a comparison between AP^{THF} and strand breaks demonstrated that the repair activity for strand breaks in these strains occurs at a lower level compared with that of the AP sites, it would imply that NDK, if involved in BER, is inefficient.

In Chapter 3, we demonstrate that RNAP bypass of both AP sites and strand breaks can cause TM *in vivo*. As depicted in Figure 5 of Chapter 3, both non-bulky base lesions, and the intermediates that result from BER, are bypassed by RNAP resulting in TM. Therefore, perturbation of any one of several steps of BER can increase the level of TM in a cell, potentially resulting in one of several biological consequences.

Transcriptional mutagenesis has many implications and potential biological consequences.

In cells that are not dividing, the contribution of TM in modification of the protein pool, and overall cellular phenotype, could overshadow mutations that are generated during replication. We envision several biological consequences of TM, both advantageous (adaptive mutagenesis) and deleterious (neurodegenerative disorders and cancer) (28).

Adaptive Mutagenesis. In single-celled organisms like bacteria, TM could be advantageous. TM could be a mechanism for adaptive mutagenesis wherein the generation of a large mutant mRNA pool and subsequent accumulation of mutant proteins could alter the cellular phenotype. If these mutant proteins then allow the

organism to temporarily escape its growth restraints, the altered phenotype could become permanent during the ensuing round of DNA replication past the lesion (29). This process is called retromutagenesis (Figure 1) because it initiates with a transcriptional event that leads to a permanent nucleotide sequence change in the DNA. Studies in bacteria and phage have shown that, for the most part, the bases that are inserted preferentially across from a lesion by RNA polymerase will be the same bases inserted opposite the lesion by DNA polymerase (30). This would ensure that the advantageous mutation that allowed the cell to escape growth restraints initially would become permanently fixed in the genome, resulting in a permanent change that is inherited by future generations.

Not only is retromutagenesis a plausible explanation for adaptive mutagenesis, but it could underlie most or all of the current hypotheses for the phenomenon. The main question that retromutagenesis addresses is how cells that are not actively proliferating and replicating can obtain the necessary mutations to escape their growth restraints. Retromutagenesis initiates entirely through a transcriptional mechanism, and thus does not require any random or infrequent replication events. Additionally, retromutagenesis allows cells to produce mutations at the RNA level first, testing the mutation without having to permanently change the DNA sequence, thus sparing the risk of deleterious mutations until it can be assured that a mutation is advantageous. In this sense, retromutagenesis and TM could be an important mechanism in the acquisition of antibiotic resistance by bacteria. Often bacteria obtain drug resistance through mutation of the gene encoding the drug target, such as the ribosomal genes (31). TM events that

allow a bacterium to overcome the drug treatment and replicate the DNA past the lesion will perpetuate in future generations, creating a population of resistant bacteria.

Cancer. While escaping growth restraints would be beneficial to bacterial cells, one could imagine that in a multi-cellular organism, such as a mammal, this process might be deleterious, as escaping growth restraints is an important step in tumor development. When quiescent cells acquire mutations in proteins involved in cell cycle progression or apoptosis, they can escape restrictions on growth and divide independently of environmental cues, contributing to tumorigenesis (32). It has been demonstrated that TM caused by 8-oxoguanine in a mutational hotspot of the *RAS* oncogene leads to a constitutively active RAS. Through subsequent activation of the MAP kinase pathway, there is a phenotypic change similar to that which has been seen in several tumors (33). We imagine several steps in which TM could contribute to the progression of cancer (Figure 2A).

It has been documented that enzymes involved in several steps of BER have been implicated in cancer development (34). The data in Chapter 3 of this thesis supports the idea that perturbation of any one of several steps of BER could have implications for cancer initiation in non-dividing cells through TM caused by BER intermediates, in addition to TM caused by unrepaired non-bulky lesions (Figure 3).

Non-bulky lesions can persist in the DNA if an N-glycosylase variant is absent, inactive, or does not bind tightly to the DNA (Figure 3). For example, one of the human N-glycosylases responsible for the processing of uracil in DNA, UNG, maps to a region of chromosome 12 that is frequently lost in gastric carcinomas (35). Also, a polymorphism in *UNG* has been found in glioblastoma, although no functional analysis

of the protein product was performed (36). UNG is involved in class switch recombination in the immunoglobulin gene clusters, and mice deleted for UNG develop B-cell lymphomas (37). OGG1 is the N-glycosylase responsible for the removal of 8-oxoguanine from DNA. OGG1 variants may increase risk of lung, esophageal, nasopharyngeal, orolaryngeal cancer and renal cancer. Additionally, these variants have decreased glycosylase activity, reduced ability to rescue the ROS-sensitivity phenotype of *mutM mutY* mutant *E. coli*, and can be found in tumors but not matched normal tissue (38-44). N-glycosylase variants have been found in many human tumors and could therefore have profound implications in the progression of cancer (34).

Abasic sites and AP-lyase-induced single strand breaks could persist in the DNA if an N-glycosylase, either monofunctional or bifunctional, fails to interact sufficiently with the AP endonuclease. Also, these lesions would persist if the AP endonuclease or end processing enzyme itself were less active, inactive, absent or did not interact appropriately with downstream BER enzymes (Figure 3). While specific human variants of N-glycosylases that are unable to interact with APE1, the human homolog of *E. coli* Nfo, have not been identified, as discussed above many of the glycosylase variants have not been fully characterized (34). In humans, there are two AP endonucleases, APE1 and APE2, and several enzymes are involved in 3′ end processing, including APE1, APE2, APTX and PNK (45). Variants in APE2, APTX and PNK have not been examined to date, but several APE1 variants have been identified in the human population (26, 46, 47). Several of these variants have not yet been evaluated for endonuclease activity, and none have been fully characterized for 3′ end processing or interaction with downstream

enzymes, so there is potential for one or several of these variants to contribute to tumor development.

While variants may encourage cancer progression through TM caused by non-bulky lesions and repair intermediates, it is important to note that perturbation of BER is not necessarily required for TM to cause cancer. The rates of repair of a lesion could easily be slow enough to allow TM to occur and force replication past the lesion before intact repair enzymes would have time to remove the lesion and prevent DNA polymerase-based mutagenesis. TM could be involved at several steps in cancer progression, and while late events in tumor progression could occur after alteration of a repair enzyme has already occurred, early events, such as TM in the *RAS* gene (33), could take place without perturbation of repair enzymes. While retromutagenesis events in humans that could lead to permanent alteration of a gene sequence, and therefore cancer initiation, would be a relatively rare event, it is important to remember that cancer is a disease of old age. Decades could be more than enough time for TM-initiated mutagenesis events to lead to cancer initiation events in at least one cell in a human body.

Neurodegeneration. Another deleterious biological consequence of TM in mammalian cells would be the death of terminally differentiated cells by accumulation of toxic proteins. One specific example is prion proteins. Prions are protein isoforms that cause transmissible neurodegenerative diseases in mammals and have implications in both public health and agriculture. In humans the diseases include Creutzfeldt–Jakob disease (CJD) and kuru, while the livestock diseases include bovine spongiform encephalopathy in cattle (also called Mad Cow disease), and scrapie in sheep (reviewed in (48)). Most known prions are self-perpetuating, amyloid-like, ordered fibrous protein

aggregates which propagate the prion state by recruiting the normal protein molecules of the same amino acid sequence (Figure 2B).

It is known that overexpression of only the prion domain of these proteins will increase the incidence of aggregation in the cell. Interestingly, in many of the prion-forming proteins, including the protein that causes disease in mammals, PrP, the prion domain is in the N-terminus while the functional domain(s) of the protein are more C-terminal (49). Therefore, if TM were to cause a nonsense mutation in the mRNA such that only the prion domain is expressed in the truncated protein, it would lead to an increased level of toxic proteins in the cell (Figure 2B). Since prions are transmissible, TM occurring in one cell could cause prions to spread to other nearby cells, perpetuating the toxic effects. In this scenario, it is not necessary for a round of DNA replication to occur and permanently fix the mutation into the genome because the aggregates can recruit both normal and mutant protein. Therefore, the lesion need only be present during the initial nucleation stage, and repair of the lesion after this stage would not prevent spread of the disease.

The results presented in this dissertation may aid in our understanding of the contributions of DNA damage to human disease. Several frequently occurring, non-bulky base lesions can cause TM when RNAP bypasses the damage and incorporates an incorrect nucleotide. Additionally, repair intermediates that result from incomplete processing of non-bulky base damage can also be bypassed by RNAP, resulting in TM. Some of this mutagenesis can be prevented by TCR, a process previously thought to only target bulky, helix-distorting DNA damage. When TCR is initiated, TM-causing lesions can be removed via BER or NER, restoring the integrity of active genes. Perturbation of

any of these DNA repair pathways could adversely affect the removal of non-bulky base damage, resulting in unfaithful transcription past damaged DNA. Through retromutagenesis, unfaithful transcription could result in deleterious consequences in humans, including cancer or neurodegenerative diseases. Therefore, these data contribute to our understanding of some of the factors that can affect human disease initiation and progression.

Future Directions

The work in this dissertation provides the basis for several future studies on the topic of DNA repair and TM. For example, it will be interesting to examine the ability of RNAP to bypass other types of repair intermediates, such as single strand breaks with different end groups (Chapter 1, Figure 4). *In vitro* studies with phage and *E. coli* RNAPs demonstrated that the ability of the enzymes to bypass different single strand breaks was variable and largely dependent upon the moieties present on the break ends, with the 3′-, 5′-phosphate gap studied in this work showing the poorest bypass efficiency (19-23). It will be interesting to determine whether different single strand break structures are bypassed differentially *in vivo*, as they were *in vitro*. Additionally, the *in vitro* studies demonstrated that single strand breaks cause single nucleotide deletions in the resultant mRNA, but this was not observed *in vivo* for the 3′-, 5′-phosphate gap (Chapter 3). Therefore it will be important to determine whether all single strand breaks are protected from frameshift mutations in the mRNA *in vivo*, or whether frameshifts result from bypass of some, but not all single strand break structures.

While it will most straightforward to examine the RNAP bypass of various strand break ends in *E. coli*, it will also be interesting to examine bypass of DNA repair intermediates such as AP sites and single strand breaks in mammalian cells. To date, the ability of mammalian RNAP II to bypass DNA repair intermediates has not been examined *in vivo*, though low levels of bypass have been observed *in vitro* (24, 50-52). The Ras system described above (33) could be an excellent assay system for these studies, not only because it utilizes a mouse embryonic fibroblast setting, but also because its readout is an important biological endpoint. Indeed, a critical future step in the study of DNA repair and TM will be to examine many more lesions for their potential contribution to human disease, be it cancer, neurodegeneration or adaptive mutagenesis in bacteria leading to antibiotic resistance.

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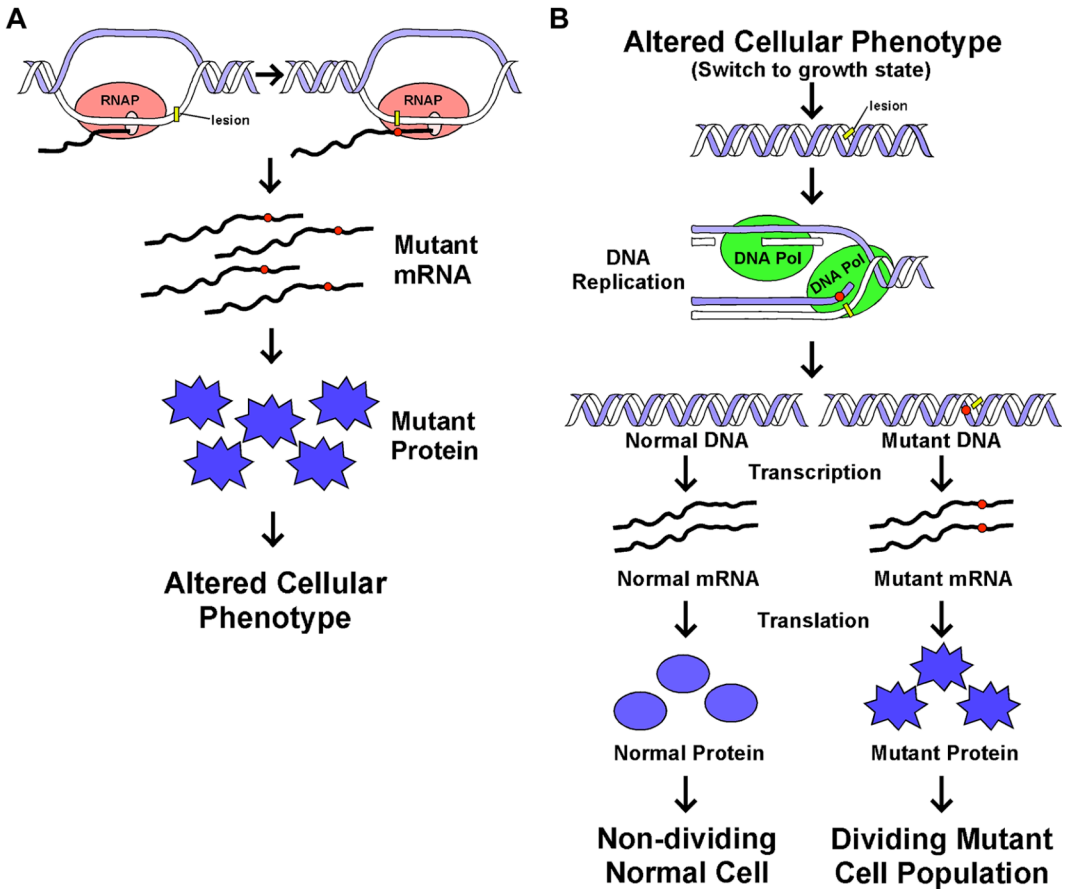

Figure 1: Retromutagenesis. If the mutant protein generated by transcriptional mutagenesis (A) alters the phenotype of the cell in such a way as to promote growth and initiate a round of replication, then the DNA lesion (if still unrepaired) will be encountered by the replication machinery (B) (top strand, lagging strand synthesis; bottom strand, leading strand synthesis). The lesion will likely cause similar miscoding during DNA synthesis, thus permanently fixing the mutation into the genome of one progeny cell. Subsequent rounds of replication in this progeny will lead to a dividing cell population harboring the mutation that conferred the growth advantage. Reproduced with permission from Saxowsky and Doetsch, Chem. Rev. (28)

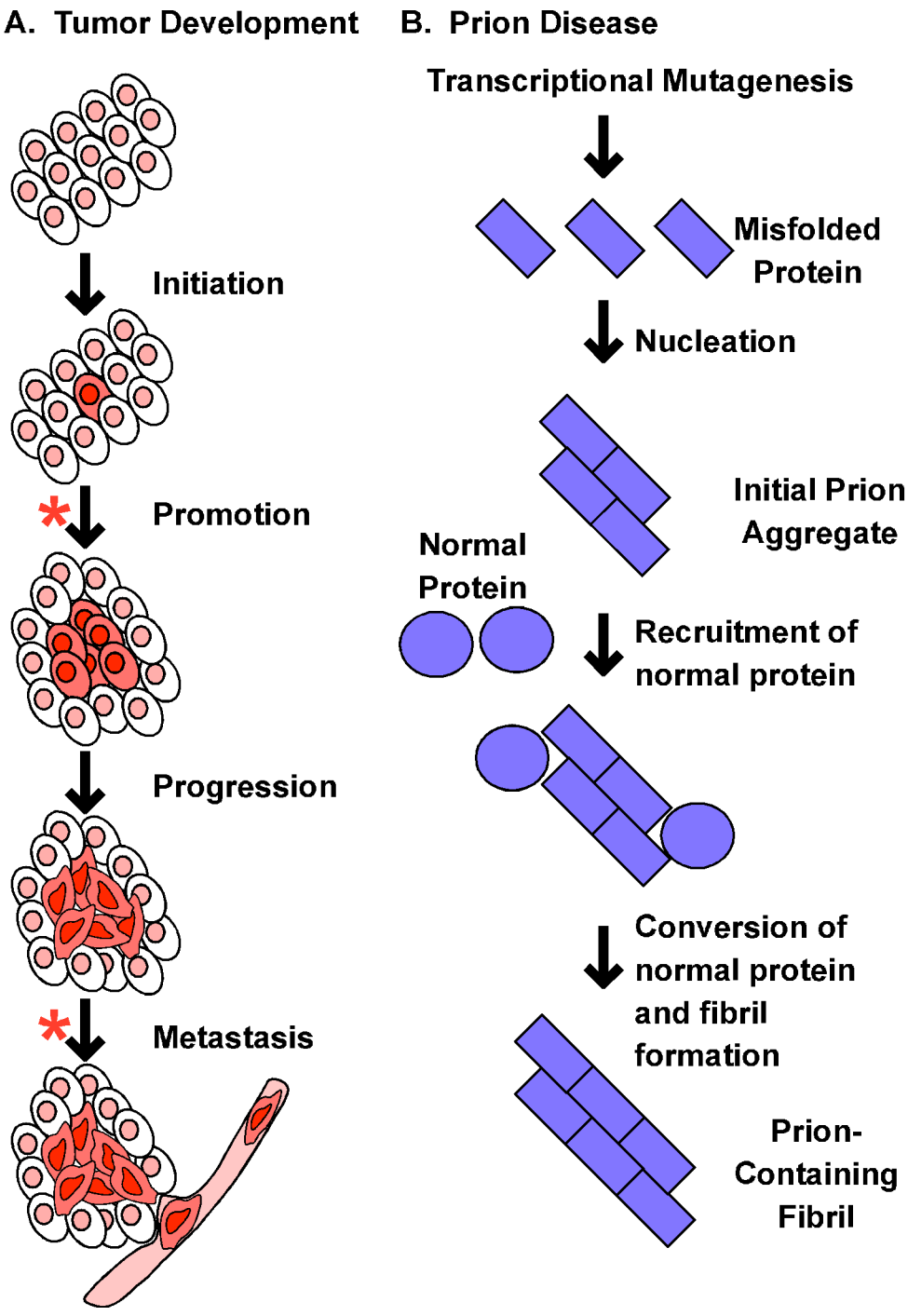

Figure 2: Transcriptional Mutagenesis in Human Disease. (A) A simplified model of tumor development. A role for TM can be envisioned at several steps, especially in tumor promotion (stimulation of cell growth) and metastasis (asterisks). (B) A model for prion formation in neurodegenerative disease. If the mutant protein generated by transcriptional mutagenesis has increased affinity for the prion conformation, it could provide fibril nucleation such that normal proteins are also depleted by way of recruitment to the fibril and conversion to the prion conformation. Reproduced with permission from Saxowsky and Doetsch, Chem. Rev. (28)

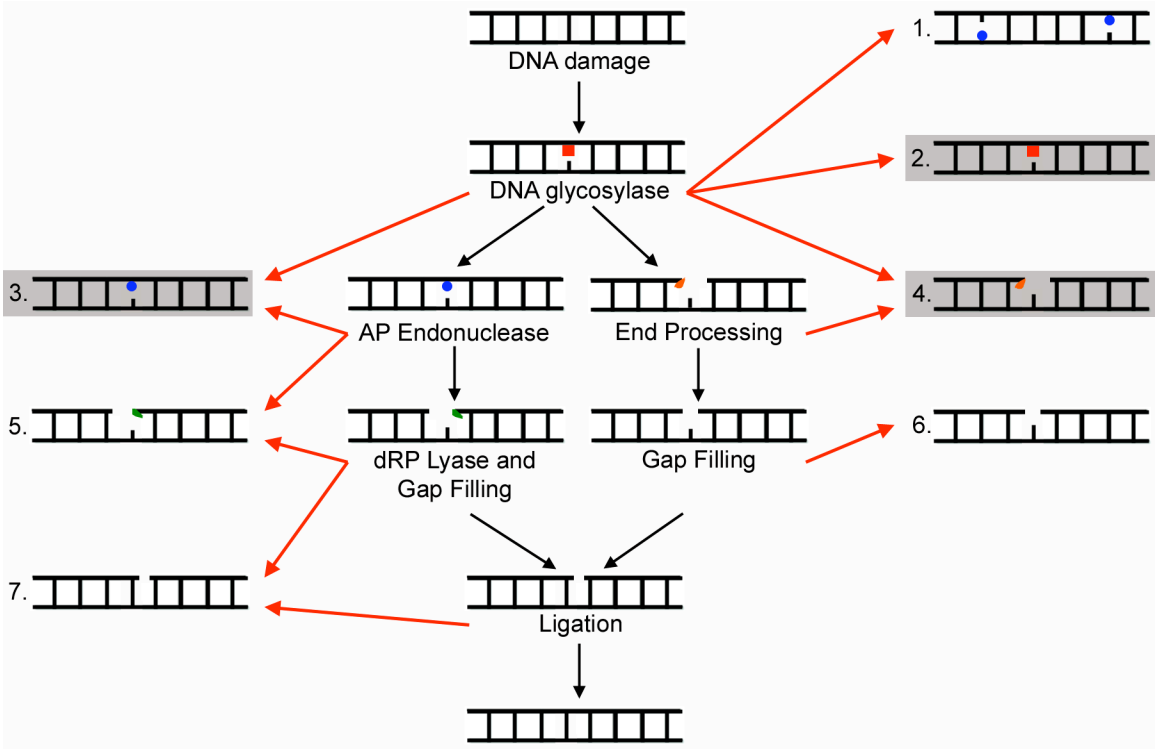

Figure 3: Possible contributions of BER variant proteins to transcriptional mutagenesis in cancer. The normal, generic short-patch BER pathway is shown in the middle. The red square denotes a non-bulky base lesion, the blue circle is an abasic site, the orange scribble is a 3′ blocking group (P or PUA), and the green scribble is a 5′ dRP moeity. The red arrows denote aberrant repair in the presence of a variant protein. On either side of the figure are DNA intermediate substrates that could result from aberrant action by BER variants. Substrates that are known to cause TM *in vivo* are boxed in grey. 1. DNA glycosylase variant recognizes normal base pairs and excises the base, resulting in an excess of abasic sites in the genome that can be subject to TM. 2. DNA glycosylase variant is inactive or does not bind tightly to DNA, resulting in TM caused by lesions left in the DNA. 3. DNA glycosylase variant is not stimulated by or does not interact efficiently with APE1, resulting in less incision. Alternatively, APE1 variant is inactive or significantly less active than the wild-type protein resulting in little incision. The resulting abasic sites in the DNA can cause TM. 4. DNA glycosylase/AP lyase does not interact efficiently with enzymes that can process 3′ blocking groups or the enzymes that process blocking groups are inactive, resulting in mutagenic bypass of 3′ blocking groups. 5. APE1 variant cannot load Pol β onto DNA, resulting in inefficient gap filling and dRP removal. Alternatively, Pol β variant has little or no dRP lyase activity, resulting in inefficient dRP removal. This structure may then be bypassed by RNAP in a mutagenic manner. 6. Pol β variant has little polymerase activity and/or interferes with the wild-type polymerase, resulting in little gap filling. This break may be bypassed by RNAP 7. Pol β variant cannot load XRCC1-LIGIIIα onto DNA, resulting in inefficient sealing of nicks. Alternatively, XRCC1-LigIIIα has little activity, resulting in a deficiency in nick sealing. RNAP may bypass this break structure as well. Figure modified and reprinted with permission from Sweasy et al., Cell Cycle, 2006 (34).